FREE MALARIAL PARASITES AND THE EFFECT OF THE MIGRATION OF THE PARASITES OF TERTIAN MALARIAL INFECTIONS.*

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PLATES 61 TO 66.

I have shown in a previous paper[1] that the malarial parasite is extracellular throughout its life cycle; that in the course of its evolution, it may destroy a number of red corpuscles, migrating from one to another; and that in the intervals between its abandonment of the degenerated remnants of the red corpuscles (corpuscular skeletons) and its subsequent attachment to the surface of fresh red corpuscles, the parasite is, for very brief periods of time, free in the blood serum. The destruction of several red corpuscles by each parasite would account for the rapidity and severity of the anemia occurring in malarial infections.[2]

The free tertian parasites pictured in this article (with the exception of figures 96 to 99, 109 to 112, 114 to 116, 121, 122, and 124) came from two cover-slip smears; one of the specimens is from a series of smears taken at intervals of approximately one half minute, the other from a series of smears taken at intervals of one half hour. No quinine had been given.

In order to make clear the relation of the free parasites to migration, I shall describe briefly the process of migration.

Various Stages in the Migration of the Tertian Malarial Parasite.—(1) Pigmented parasites on decolorized red corpuscles or on corpuscular skeletons (granular degenerated remnants of red corpus-

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[1] Rowley-Lawson, M., *Arch. Int. Med.*, 1912, ix, 420.

[2] Marchiafava and Bignami (1) write: "In no other infection is anaemia produced with the same rapidity and to the same extent as in malaria."

cles); (2) pigmented parasites in the act of abandoning the decolorized or granular degenerated red corpuscles; (3) pigmented parasites, free in the blood serum; (4) pigmented parasites in the same stages of development as the parasites described under stages 1, 2, and 3, but attached to red corpuscles, the hemoglobin of which appears to be as yet unaltered.

The Rapidity with Which Migration May Take Place.—I believe the process of migration to be very rapid. The young unpigmented parasites usually appear almost immediately attached to red corpuscles. Thayer (2), in writing of these fresh segments, states: "They appear in the red corpuscles simultaneously with, or shortly after the appearance of the sporulating bodies." I believe that the free pigmented parasites in migration also attach themselves almost immediately to fresh red corpuscles. This belief is supported by the following facts: (a) all the above stages in migration may be present in one smear; and (b) only two successive smears, from the series of 200 smears taken at one half minute intervals, showed what I interpret as stages in migration.

Parasites Free in the Blood Serum.—At times there may be seen free in the blood serum segments resulting from a recent segmentation, and pigmented parasites in several stages of development and in various phases: (a) presegmenting (figures 110 and 111); (b) segmenting (figure 109); (c) individual segments (figures 36 and 96); (d) ring-form (figures 1 to 14, 48, 57, and 67); (e) ameboid (figures 62 to 64, 69, and 70); (f) compact (figures 16 to 35, 38 to 40, 47, 49, 51, 114 to 120, and 126); (g) with protoplasmic pseudopodia (figures 37, 41 to 46, 50, 52 to 56, 58 to 61, 65, 66, 68, 71 to 94, 95 to 102, 104 to 108, 112, 113, 122, 123, 125, 127, 128); (h) with flagella (figure 113).

Presegmenting Parasites.—This type of parasite is so often found free, that I believe the parasite must always migrate in this stage of development.

Segmenting Parasites.—In these instances the smear must have been taken just as the corpuscle was destroyed, and before the individual segments had time to take up their separate existence.

Individual Segments.—It is not uncommon to see the parasites free in this stage of development; but it is extremely rare to find one of them with a pseudopodium (figure 96).

Ring-Form Parasites.—These parasites, on destroying the red corpuscles to which they were attached, have been freed in the form which they had assumed while surrounding a corpuscular mound (figure 15).

Ameboid Parasites.—These parasites have either been freed in this form after having destroyed the corpuscle to which they were attached, or have assumed it in preparation for a new attachment. Parasites in this phase are often difficult to distinguish from parasites with protoplasmic pseudopodia.

Compact Parasites, or Parasites Which Have Contracted Their Protoplasm into a Discoid or Spheroidal Form.—These are the types of free parasites most commonly seen both in fresh blood preparations and in stained specimens, and the compact form is, I believe, the normal resting form of the parasite when living, and that usually observed when the parasite is dead. It is in this form that the parasite is generally found in the blood of cinchonized patients, and in smears from the cadaver.

In referring to this form of the living parasite, Laveran (3) states that spherical bodies are at times free in the blood serum, and he pictures them in several stages of development. Mannaberg (4) writes: "The perfectly globular form is first assumed by the parasite when it has escaped out of the red blood corpuscle." And Marchiafava and Bignami (5), in referring to these free compact parasites, state: "We would call attention to the presence of pigmented spherules of varying sizes, which are almost always found free in the blood of a tertian patient. They are of importance in diagnosis, because sometimes they exist alone [migration?], the most careful examination failing to reveal any endoglobular parasitic bodies. These spherules are composed of a hyaline substance with small granules of freely moving pigment, and possess so characteristic an appearance to the expert eye that they quite suffice to a diagnosis of malarial tertian. For the greater part they are merely parasites which have escaped from the red corpuscle."

I have not made an exhaustive study of specimens of blood taken after the patient had been given quinine; but such smears as I have studied showed an almost complete absence of parasites. I have seen free compact parasites after the use of quinine; but I do not

believe that these parasites were in migration, for they showed no such uniformity in their stages of development in any one smear as healthy parasites in migration ordinarily do, nor did they take the stain as vividly as did the healthier parasites. I believe that it was the action of the quinine that caused these parasites to abandon the red corpuscles and assume the compact form. Marchiafava and Bignami (6) state that quinine may cause certain parasites to abandon the red corpuscles, and that (7) ring-form bodies disappear from malarial blood which has been strongly cinchonized. Romanowsky (8) describes "'quinine forms' in which the clear zone of the nucleus was wanting [the corpuscular area], this structure fading insensibly into the body of the parasite"; and Golgi (9) claims that after quinine, the body of the tertian parasite becomes round and motionless. I believe that in these instances the parasites were either sick or dead.

In smears from the cadaver I have seen free parasites only in the compact form. Indeed Marchiafava and Bignami (10) go so far as to assert that "in the cadaver we do not see annular bodies; but only, as a rule, immotile discoid or spherical micrococciform bodies." In these instances I believe that the compact form is the result of the death of the parasites.

Parasites with Protoplasmic Pseudopodia.—These are parasites in varying stages of development, with attaching pseudopodia which are derived from the cytoplasm of the parasite. These pseudopodia may vary both in length and in number, and may or may not contain pigment granules. I believe that the pseudopodia are used by the parasites to secure their prey, as well as for the purpose of attachment to the red corpuscle. These attaching processes must be sharply distinguished from flagella, which arise from the chromatin substance of the parasites. Free parasites with pseudopodia are only rarely seen in stained smears from the circulating blood. They are most commonly thrown out when the parasite is free from the corpuscle; but very rarely one or more of the filaments may be seen going off beyond the periphery of the degenerated red corpuscle to which the parasite is still attached (figures 103 and 125). Dock (11) has also described and pictured the pseudopodium of a parasite going off beyond the periphery of the red corpuscle to which the parasite was attached.

Celli and Guarnieri, and later also Plehn (12), believed that they saw spores in the blood serum which "swarmed by the means of flagella" (figure 96 shows a spore with a flagellum), and Thayer (13) has observed that the "small hyaline bodies may sometimes be followed for some little distance from their original segmenting form. Under these circumstances they may show a slight dancing to-and-fro movement which suggests the possible existence of flagella."

Parasites with Flagella.—These parasites are microgametocytes in the sexual phase, the flagella arising from the chromatin substance of the parasite. In stained specimens, as a rule, the sexual flagella have a more curled appearance than do the protoplasmic attaching processes. In my experience it is rare to find pigment in connection with flagella. Flagellation may occur either when the parasite is free from the red corpuscle, or when it is still attached to it. Preflagellating parasites are occasionally found free, and may be seen with or without attaching filaments (figure 112, with attaching pseudopodia). This form of parasite is usually smaller than the segmenting bodies in the same infection, is deeply stained, and contains more chromatin in proportion to its protoplasm.

Sexual flagellating parasites are only occasionally found in stained smears from the circulating blood, but now and then a few examples may be seen. Rarely one is fortunate enough to find the complete sexual cycle.[3] I have found actively sexual parasites in all types of malaria, but only in cases showing very heavy infections. It seems to me that the term flagellata is a misnomer when applied to parasites in the sexual phase; but as it is the term which is generally accepted as applying to parasites in this phase, I continue to use it.

Sexual flagellating parasites should not be confused with parasites extruding protoplasmic pseudopodia. I believe that many of the so called flagellating bodies seen in fresh blood preparations are not parasites in the sexual phase, but parasites which, on destroying the corpuscle to which they were attached and becoming free, proceed to throw out protoplasmic processes for the purpose of capturing and reattaching themselves to other red corpuscles. It has often

[3] Rowley-Lawson, M., *Jour. Exper. Med.*, 1911, xiii, 263.

been noted that the red corpuscle is practically destroyed before the parasite abandons it and then proceeds to flagellate. Why should the corpuscle be destroyed before the parasite flagellates, if this flagellation is always a sexual phase? Does the parasite time its destruction of a red corpuscle to correspond with, or nearly with, the developmental stage of a sexual flagellation? I have stained some of these so called flagellating bodies and found the filaments composed of protoplasm only, and several observers have stated that the flagella observed by them appear to be protoplasmic in nature and that the nucleus does not take part in the process of flagellation. Antolisei (14) believes flagellation to be a degenerative process; the flagella he describes as sarcodic prolongations of the protoplasm. Grassi and Feletti (15) state that the nucleus does not take part in the process of flagellation, neither dividing nor entering into the flagella. Marchiafava and Bignami (16) found that "there are some flagellata in which the filaments do not contain any chromatin; but are composed of protoplasm alone. And others in which there may be one or two filaments provided with chromatin, the others being formed of protoplasm." Figure 113 shows a free flagellating parasite, with one filament composed of chromatin (a); the other filaments (o) are formed from the cytoplasm of the parasite, and pigment granules may be seen in connection with them.

Sakharoff (17) describes what I believe to be genuine sexual flagellating parasites. "The process of the formation of the flagellate bodies, consists in a perversion of the karyokinetic nuclear division, in a breaking up of the nucleus into the chromatin filaments, and in the escape of these from the parasite; these filaments which are in lively motion represent the flagella." Sakharoff believed this process of flagellation to be a degenerative one.

Malarial parasites in various stages of development have been noted and pictured free in the blood serum by so many observers, that it is surprising that no rational explanation has been offered to account for them. Laveran (18) constantly refers to them, stating that they may "often be found in the blood in a free state, and that at all periods of their development." Mannaberg (19) writes: "The spores which have become free . . . are often to be demon-

strated in enormous numbers. . . . The amoeboid bodies too, which are developed further than their spore-forming stage, are not infrequently to be found free in the liquor sanguinis, fully retaining their structure." Thayer and Hewetson (20) state: "Occasional pigmented bodies may be found outside the red corpuscles, free in the plasma. Some of these represent full-grown bodies which have destroyed the surrounding corpuscle, while in other instances, the half-grown forms may actually leave their host." Ewing (21), in his monograph on malarial parasites, states that Celli and Guarnieri and Ziemann have depicted free parasites and that he himself has seen them in fresh blood preparations; but he believes that they are extremely rare in circulating blood. He writes: "In preparations of fresh blood, parasites so frequently pass from the cell into the plasma that it may be doubted if any accurate estimation of the number of extra-cellular bodies in the circulating blood can be obtained by this method of examination"; and Ruge (22) has pictured free parasites in several stages of development.

Bass and Johns (23) claim that the malarial parasites "cannot live for even a few minutes free in the serum." Of course this statement must apply only to parasites in artificial media or under very abnormal conditions, for all observers know that parasites are seen to remain in motion and apparently alive for many minutes free in the serum. Laveran (24) has noted that they may remain alive for half or three quarters of an hour. Marchiafava and Bignami (25) have noted that in flagellated bodies the movements of the pigment granules within the pigmented body "may continue for hours." Thayer and Hewetson (26) note that motions may be observed in flagellate bodies sometimes for as long as half an hour, the flagellating bodies being free; for they (27) state, "We have never seen the appearance of flagella in bodies still contained in the red blood corpuscles." Schaudinn (28) claimed to have watched under the microscope the "entrance into a red corpuscle" of a young parasite resulting from a segmentation, and I have frequently seen free parasites remain alive for over half an hour. The segments from a sporulating body as well as the parasites which are injected by the infecting mosquito must also be free in the blood serum for a certain length of time.

If all malarial parasites are, as I believe, attached to the external surface of the red corpuscles,[4] they must constantly be exposed to the action of the patient's serum. Stained specimens seem to show that the free parasites in migration do not remain long free, but attach themselves almost immediately to fresh red corpuscles; and if we stop to consider how short the life of the individual parasite is, even the brief periods of time when it is free in the blood serum may be comparatively long. Hence one would expect, under normal conditions for the parasite, what to us seems an immediate attachment.

The Effect of Migration, the Progressive Loss of Red Blood Corpuscles in the Intervals between Paroxysms in Tertian Malarial Infections.—Several years ago, while trying to estimate the degree of anemia in certain cases of tertian malaria showing very heavy infections, the late Dr. J. H. Donoghue of Boston and I took red counts at certain intervals, from one segmentation period to seven to eight hours after the following segmentation period, before giving quinine. The segmentation period was determined from stained specimens and not estimated from the time of the chill. We found that the anemia progressed between the paroxysms, that the greatest fall in the red corpuscle count occurred, not, as we had expected, soon after the segmentation of the parasites with the resulting destruction of red corpuscles to which they had been attached, but from six to seven hours later. Unfortunately we did not control each intermediate count of red corpuscles with a stained specimen. Later, in going over the stained specimens from these cases, I found in some of the smears certain free parasites, all in a few definite uniform stages of development. I did not at first connect these free parasites with the progress of the anemia between the paroxysms; but later I interpreted them as migratory parasites capable of destroying more than one red corpuscle. In these cases blood regeneration began as soon as quinine was given.

The maximum fall in the red count several hours after each segmentation period is due, I believe, not only to the loss of red corpuscles resulting from the segmentation of the parasites, but also to the subsequent and further destruction of corpuscles by the new

[4] Rowley-Lawson, M., *Jour. Exper. Med.*, 1913, xvii, 324.

group of parasites. Kelsch (29) observed the progress of anemia between the paroxysms, and stated (30) that in a robust individual, in the course of one day, the number of red corpuscles may go from normal to 1,000,000 per cubic millimeter. Dionisi (31) observed a continuous loss of red corpuscles during the afebrile interval, and Ewing (32) states that the anemia may progress during afebrile periods. I believe that quinine must be kept up for longer periods than is usual in order to insure a complete elimination of the parasites, otherwise we may be confronted with relapses. Although the malarial parasites may show a variable resistance to quinine, I do not believe that there is any stage in their development or phase in their existence when they are absolutely impervious to persistent and long continued treatment.

SUMMARY.

1. The malarial parasite is extracellular throughout its life cycle and migrates from red corpuscle to red corpuscle destroying each before it abandons it; in the brief intervals between, the parasite is free in the blood serum; it does not remain long free, but almost immediately attaches itself to another red corpuscle by means of delicate pseudopodia.

2. The compact form of the tertian parasite is the type of free parasite most often observed; in this form the parasite may be seen not only in migration, but after quinine and in the cadaver. I believe the compact form to be the normal resting form of the parasite, all other forms being assumed in order to secure attachment and to obtain food.

3. Care must be taken not to confound free parasites having protoplasmic pseudopodia ready for attachment with the sexual flagellating parasites, whose flagella are composed of chromatin.

4. The malarial parasite can live for some time free in the blood serum, though under normal conditions there is no reason why it should remain free for any length of time, and there are certain periods in the life of the parasite when it must be admitted that it is free from the corpuscle and survives. If the parasite is, as I believe, attached to the external surface of the red corpuscle, it is constantly exposed to the action of the patients' serum.

5. The destruction of more than one red corpuscle by each parasite would readily account for the severe and early anemia occurring in malarial infections.

6. Long continued treatment with quinine will eventually cause the death of all malarial parasites.

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EXPLANATION OF PLATES.

PLATE 61.

FREE TERTIAN MALARIAL PARASITES.

Magnification, × 1,725.

FIGS. 1 to 14. Pigmented ring-form parasites freed in the form which they had assumed when encircling corpuscular mounds. In figure 4 a very delicate pseudopodium may be seen at x. In figure 13 the periphery of the adjacent red corpuscle shows through the opening of the ring.

FIG. 15. The ring-form is seen encircling the corpuscular mound of a red corpuscle.

FIGS. 16 to 35. Various pigmented compact parasites with contracted protoplasm. Figure 20 illustrates what I believe to be a microgametocyte.

PLATE 62.

FREE TERTIAN MALARIAL PARASITES.

Magnification, × 1,725.

FIG. 36. Two segments resulting from a recent sporulating body.

FIG. 37. A young pigmented parasite with an attaching pseudopodium.

FIGS. 38 and 39. Two young pigmented parasites with contracted protoplasm.

FIG. 40. A young unpigmented parasite which has, I believe, been freed prematurely from a red corpuscle, probably by a parasite of more advanced growth destroying the corpuscle.

FIGS. 41 to 45. Young pigmented parasites with protoplasmic pseudopodia out for attachment to fresh red corpuscles. In figure 44 the parasite appears to be already attaching itself to the red corpuscle.

FIGS. 46 to 70. Pigmented parasites, ring-formed, ameboid, compact, and with protoplasmic pseudopodia.

PLATE 63.

FREE TERTIAN MALARIAL PARASITES.

Magnification, × 1,765.

FIGS. 71 to 94. Pigmented parasites with pseudopodia arising from the cytoplasm of the parasites.

PLATE 64.

FREE TERTIAN MALARIAL PARASITES.

Magnification, × 1,725.

FIGS. 95 to 108. Parasites with pseudopodia arising from the cytoplasm of the parasites. Pigment granules may be seen in connection with many of the pseudopodia.

FIG. 96. A young segment with a pseudopodium. It is rare to find segments with pseudopodia in stained specimens.

FIG. 98. The pseudopodium is seen to be perfect in form and its length is twice the diameter of a red corpuscle.

FIG. 103. The pseudopodium of the parasite is seen extending beyond the periphery of the dehemoglobinized red corpuscle to which the parasite is still attached.

PLATE 65.

FREE TERTIAN MALARIAL PARASITES.

Magnification, × 1,725.

FIG. 109. A segmented parasite free from a red corpuscle.

FIGS. 110 and 111. Presegmenting parasites free from red corpuscles.

FIG. 112. A preflagellating parasite (microgametocyte) free from the red corpuscle, with protoplasmic pseudopodia at x. Note that the parasite has been freed in the form which it had assumed when surrounding a corpuscular mound. Compare this parasite with the presegmenting parasites and note that it contains more chromatin in proportion to its cytoplasm than do the presegmenting bodies.

FIG. 113. A sexual flagellating parasite (microgametocyte) with the flagellum composed of chromatin at a, and pseudopodia from the cytoplasm at o. Granules of pigment may be seen in connection with the pseudopodia.

FIG. 114. This shows what I interpret to be a microgametocyte free from the red corpuscle. Careful examination will show what appear to be pseudopodia just starting out at the periphery of the parasite on the left.

FIGS. 115 to 120. Large free pigmented parasites. Note that the chromatin in figures 117 to 120 is very palely stained. I believe these parasites to be unpregnated adult macrogametes.

FIG. 121. An adult pigmented parasite almost freed from a red corpuscle showing advanced granular degeneration.

FIG. 122. An adult pigmented and well stained parasite which was probably about to abandon the degenerated remnant of the red corpuscle to which it was attached before the spreading of the smear. The process of smearing the blood probably separated the parasite from the remnant.

FIG. 123. An adult pigmented parasite similar to those shown in figures 117 to 120, but with a pseudopodium, suggesting that the parasite is preparing for another attachment.

FIG. 124. Two parasites, an adult pigmented and a young unpigmented parasite, still attached to a red corpuscle showing advanced granular degeneration. I believe that the adult parasite is preparing to migrate and in doing so it will also free the young parasite, probably prematurely.

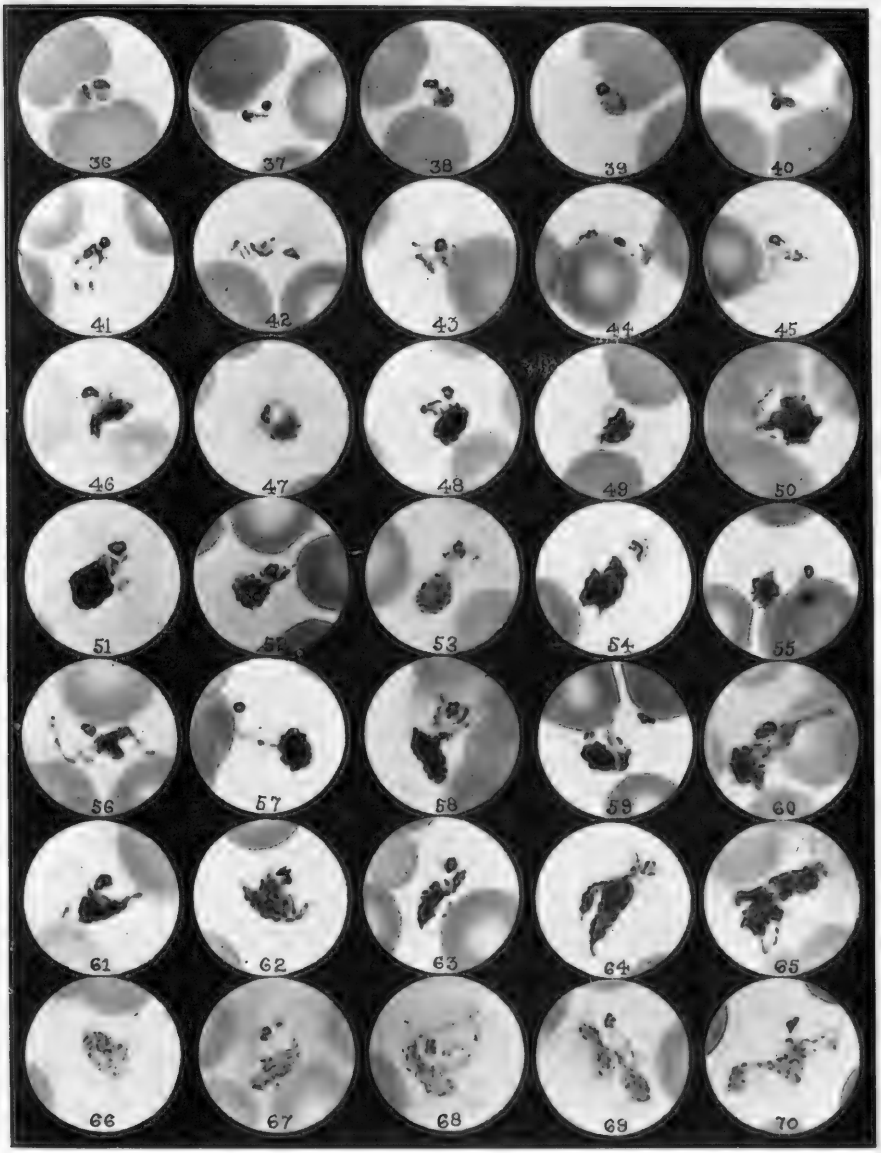
FIG. 125. A pigmented parasite preparing to migrate from a degenerated red corpuscle. Note the pseudopodium extending beyond the periphery of the red corpuscle.

FIG. 126. A young parasite which appears to be only just freed from the remnant of a degenerated red corpuscle, shown just below the parasite.

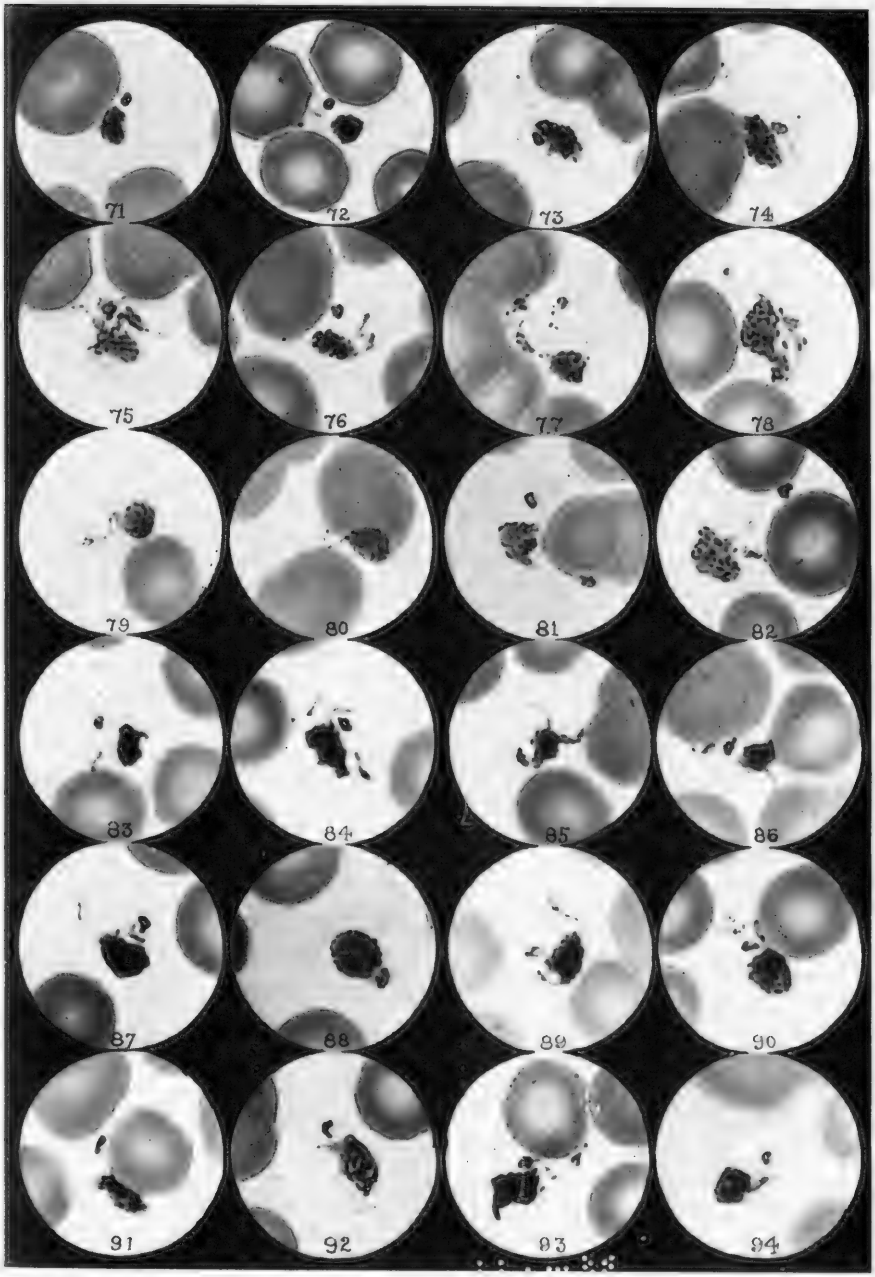
FIG. 127. A free parasite with protoplasmic pseudopodia.

FIG. 128. A free pigmented parasite with protoplasmic pseudopodia.

(Lawson: Free Malarial Parasites.)



(Lawson: Free Malarial Parasites.)



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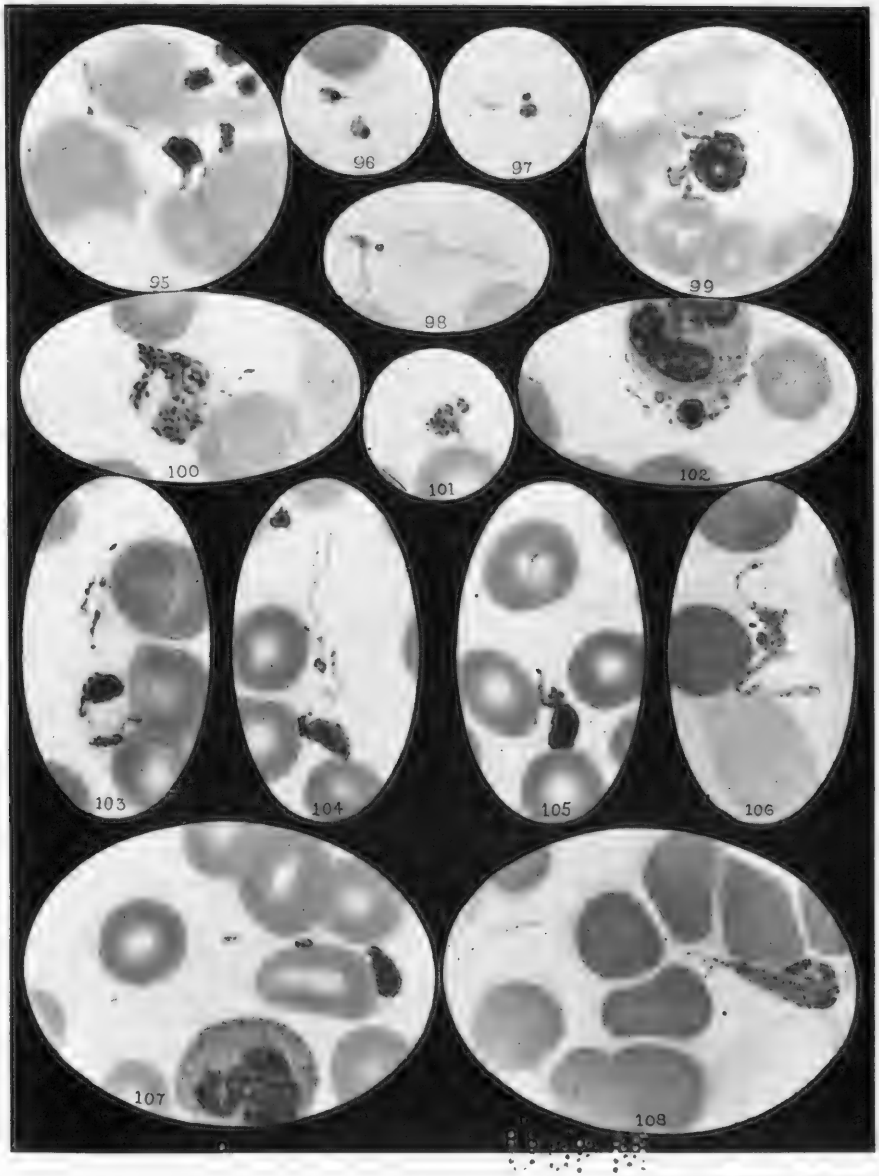

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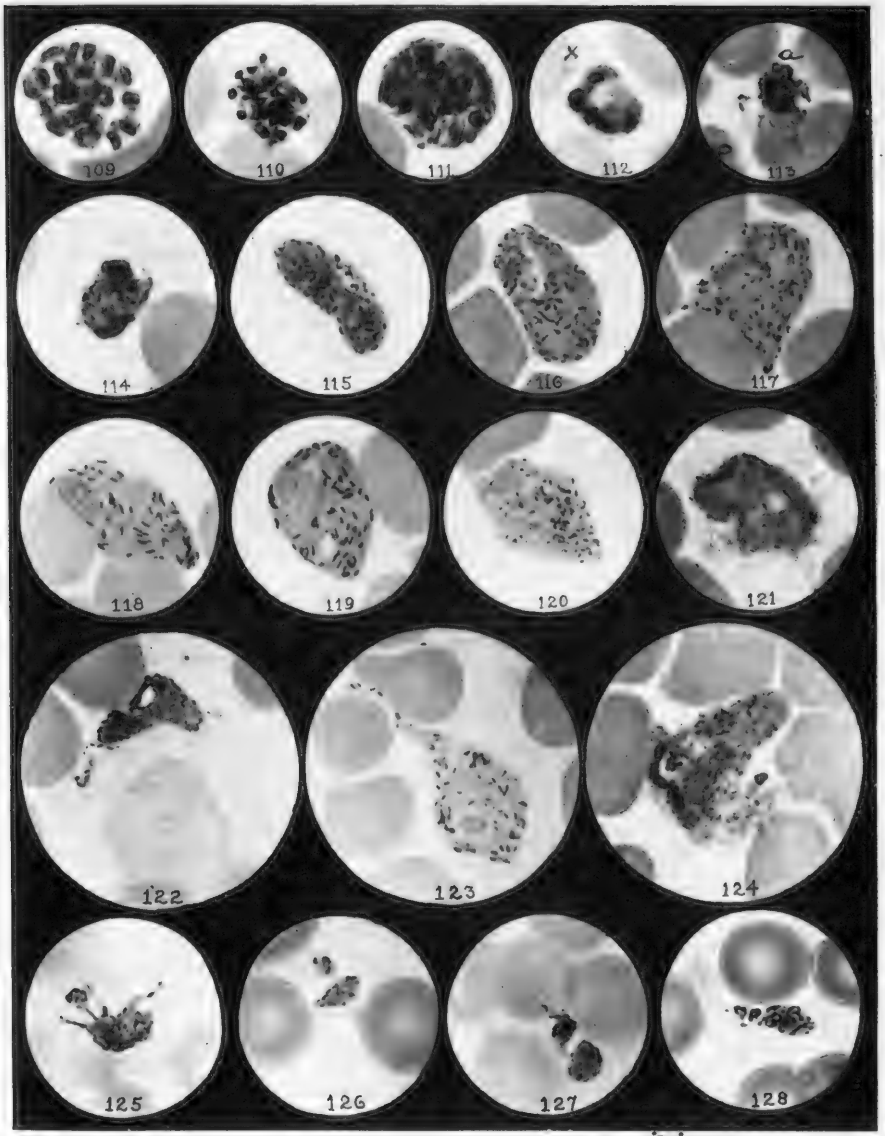

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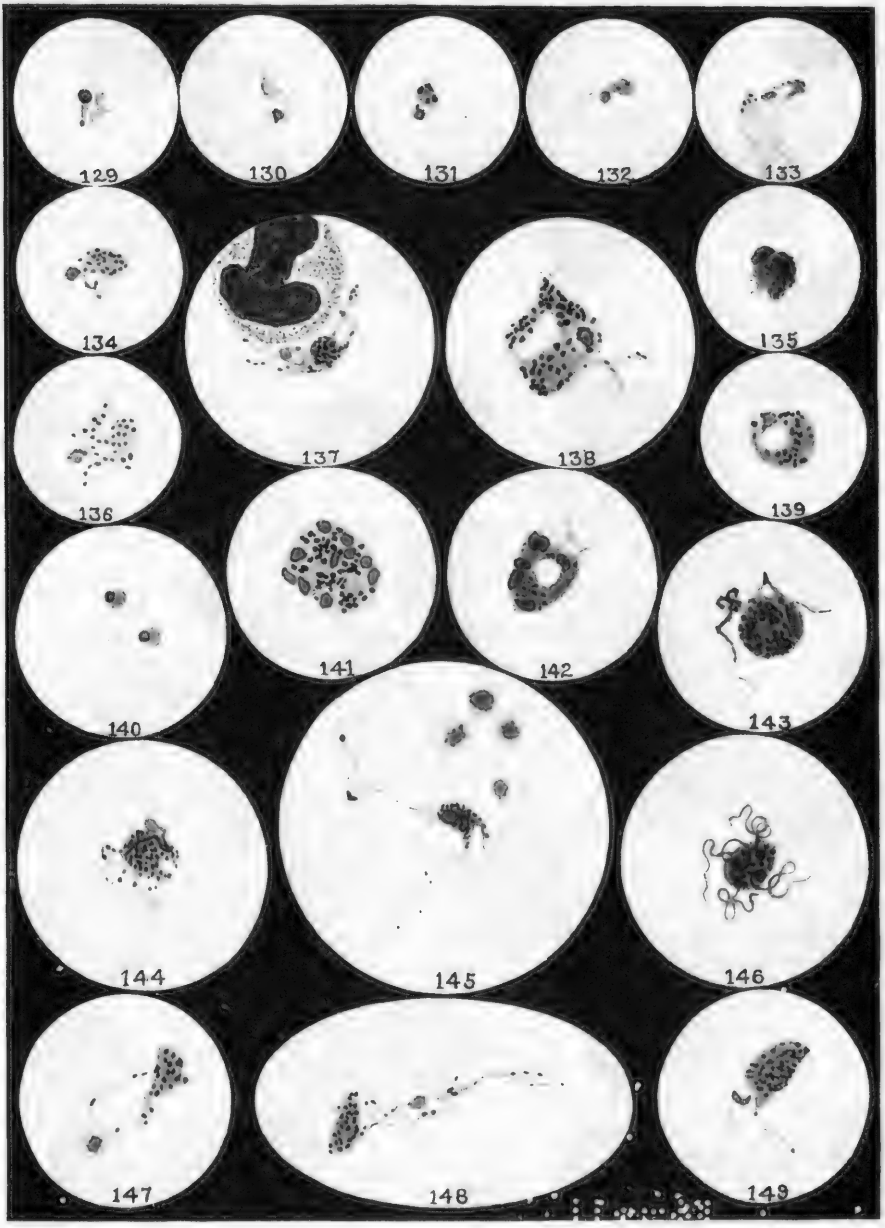

(Lawson: Free Malarial Parasites.)

PLATE 66.

FREE TERTIAN MALARIAL PARASITES.

FIGS. 129 to 133. (Correspond to figures 43, 42, 45, 37, and 44.) Young pigmented parasites with protoplasmic pseudopodia. In figure 133 the parasite appears to be attaching itself to the red corpuscle.

FIG. 134. (Corresponds to figure 48.) A pigmented parasite, freed with the pseudopodia in the form of a loop, which had been used to encircle a corpuscular mound.

FIG. 135. (Corresponds to figure 20.) A compact pigmented parasite.

FIG. 136. (Corresponds to figure 75.) A pigmented parasite with pseudopodia arising from the cytoplasm of the parasite.

FIG. 137. (Corresponds to figure 102.) A pigmented parasite with pseudopodia arising from the cytoplasm of the parasite.

FIG. 138. (Corresponds to figure 100.) A pigmented parasite with pseudopodia arising from the cytoplasm of the parasite.

FIG. 139. (Corresponds to figure 7.) A pigmented ring-form parasite, freed in the form which it had assumed when encircling a corpuscular mound.

FIG. 140. (Corresponds to figure 96.) A segment resulting from a recent sporulation, with a pseudopodium. It is rare to find one of these young parasites with a pseudopodium.

FIG. 141. A free presegmenting parasite.

FIG. 142. (Corresponds to figure 112.) A preflagellating parasite (microgametocyte) with pseudopodia. The parasite has been freed in the ring-form which it had assumed when encircling a corpuscular mound. Compare this parasite with the presegmenting body.

FIG. 143. (Corresponds to figure 99.) A pigmented parasite with pseudopodia arising from the cytoplasm of the parasite.

FIG. 144. (Corresponds to figure 113.) A sexual flagellating parasite (microgametocyte) with flagellum composed of chromatin substance, and pseudopodia (attaching processes) composed of the cytoplasm of the parasite.

FIG. 145. (Corresponds to figure 95.) A pigmented parasite with pseudopodia (attaching processes) arising from the cytoplasm of the parasite. Pigment granules can be seen in connection with some of the filaments. The other bodies seen in the picture are blood plates.

FIG. 146. A sexual flagellating parasite (microgametocyte), the flagella being derived from the chromatin substance of the parasite. Compare this parasite with the parasites with attaching pseudopodia. The attaching pseudopodia arise always from the cytoplasm of the parasite.

FIG. 147. A pigmented parasite with the pseudopodia in the form of a loop, which formerly was used to surround a corpuscular mound.

FIG. 148. (Corresponds to figure 104.) A pigmented parasite with attaching pseudopodia arising from the cytoplasm of the parasite.

FIG. 149. (Corresponds to figure 92.) Pigmented parasite with attaching pseudopodia arising from the cytoplasm of the parasite.

THE ADRENALIN INDEX OF THE SUPRARENAL GLANDS IN HEALTH AND DISEASE.*

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During the past two years three methods for the quantitative estimation of adrenalin have appeared. They are accurate and sensitive to minute quantities of adrenalin, much more so than any quantitative methods hitherto used. The method of Elliott consists in the blood pressure response of a decerebrate cat to intravenous injection, standard adrenalin solutions being used as controls. The method of Folin consists of a colorimetric reaction of adrenalin with a phosphotungstic acid reagent, the standard being a pure uric acid solution. The method of Seidell consists of a colorimetric reaction of adrenalin with manganese dioxide, with adrenalin and gland extract as a control. Seidell's method appeared after this series of observations had been completed and we cannot therefore report on its usefulness in this type of work.

The chromaffin tissue is thought to have an intimate association with adrenalin production and storage. This tissue gives a deep brown color when treated with a solution of potassium bichromate, and various studies dealing with this substance have been published.

Vincent reports a study of this chromaphil substance in many animals, particularly the dog and cat. He notes that the chromaphil substance is found in considerable abundance in the dog outside of the adrenal medulla. The abdominal chromaphil body is situated just in front of the aorta, usually below the level of the adrenals. It is incapable of maintaining life in the absence of the two adrenals. An extract of this tissue can cause a rise in blood pressure like that produced by an extract of adrenal medulla. He states with reason that this body must be taken into consideration in all interpretations of experiments in which dogs are used.

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Schur and Wiesel claim that prolonged narcosis with chloroform or ether in rabbits may cause disappearance of the adrenal chromaffin, followed by a rapid return to normal in about twelve hours. They note hypoplasia of the chromaffin tissue of the adrenal medulla in status lymphaticus. Schwarzwald confirms this last observation, but not the observations on animals after anesthesia. Hornowski claims that in man and animals one may note a quantitative diminution of the adrenal chromaffin, depending upon the duration of anesthesia. He suggests that shock may depend in part on this depletion of the medulla chromaffin, which is concerned in the secretion of adrenalin.

Borberg has done an extensive series of experiments to show fluctuations in the chromaffin tissue under various experimental conditions. He used the chromate reaction and states that the chromaffin tissue coloration and adrenalin content vary with absolute parallelism, the one perhaps being the parent substance of the other. Inanition and asphyxia give a loss of chromaffin reaction; anemia may show a normal reaction; and intestinal obstruction shows a paler one. Chloroform anesthesia of short duration gives no change, but long anesthesia (three to seven hours) gives a marked weakening of the chromaffin staining. Ether or urethane anesthesia causes no change. Phosphorus poisoning gives a slight lowering of the chromaffin staining. He finds no indication by this method of hyperplasia of one adrenal after removal of the other gland. There is no reasonable doubt that the methods used in our work are much more delicate than the chromaffin reaction. If it is justifiable to argue concerning the adrenalin content from the density of the chromaffin reaction, surely one can reverse the process and assume that a more accurate estimation of adrenalin may give evidence concerning the depletion of the chromaffin tissue.

Elliott states that the chromaffin tissue diminishes in amount as the adrenalin content falls, but that no accurate determination is possible. His experiments deal chiefly with the adrenal glands of cats under various experimental conditions. He states that anesthesia by chloroform, ether, or urethane will cause a drop in the adrenalin content of the glands. Stimulation of sensory nerves or injury to the brain will cause a definite fall in the adrenalin content of the glands. He believes that drugs, stimuli, and trauma act through the central nervous system and that the impulses travel along the splanchnics.

METHOD.

A great many observations have been discarded because of inexplicable variations or slips in technique. This applies especially to the physiological method, as it will be found that some cats are refractory and give variable reactions to injections.

The glands from the human cases were removed at autopsy, and if not to be used immediately were placed on ice. They were usually cleaned and dissected carefully within a few hours of the autopsy, and the extract was made and tested. The adrenals were weighed and in many instances separate extracts made from the two glands (tables I to XVII).

The glands obtained from dogs were always fresh and removed at the end of an experiment or a few minutes after death. When not tested at once, the glands were wrapped in omental fat and placed on ice, but unless indicated in

the tables the extracts were made shortly after death. Experiments were done to indicate the influence of this preservation on ice and the rapidity of deterioration. It was found that gland *Brei* kept on ice for twenty-four to forty-eight hours would rarely show a diminution of 5 per cent. in adrenalin.

After dissection and weighing the glands were cut up and ground with sand to a uniform paste. This is a time-consuming process, but the determinations will not be uniform and dependable unless it is thoroughly carried out. The paste is then made up to a 20 per cent. solution with 0.9 per cent. salt solution, ground, and mixed thoroughly. After the sand has settled, the milky fluid is decanted and used as follows:

A. 5 c.c. of the 20 per cent. extract are combined with 5 c.c. of N/5 hydrochloric acid and heated to boiling. Sodium acetate (10 per cent.) is added drop by drop to the boiling solution until coagulation is complete. The mixture is diluted to 50 c.c. and filtered through paper giving a water-clear solution which corresponds to the solution used by Folin. Of this clear extract 10 c.c. are pipetted into a 100 c.c. volumetric flask, and 2 c.c. of the uric acid reagent and 20 c.c. of a saturated solution of sodium carbonate are added. The solution is shaken, made up to the mark, and after standing a few minutes is read against the standard solution. It is often necessary to use more than 10 c.c. of the gland filtrate to give enough color for accurate reading.

The standard uric acid solution is made by placing 1 c.c. of a fresh solution of uric acid containing 1 mg. of Kahlbaum uric acid in a 100 c.c. volumetric flask. The uric acid reagent and sodium carbonate are added as before and the solution is made up to the mark. The readings must be made promptly in a standard colorimeter as the colors fade. Folin, Cannon, and Denis have determined that pure adrenalin gives three times as intense a color as pure uric acid, so that by a simple calculation the amount of adrenalin in milligrams per gram of gland may be calculated.

B. Another portion of gland extract (20 per cent.) is diluted for use in the physiological test. With normal human gland extract a 1 per cent. solution is most satisfactory, but at times further dilution may be necessary, or in other cases a 10 per cent. extract must be used to elicit a satisfactory reaction. With canine gland extract it may be necessary to dilute to 0.2 per cent., so that a 1 c.c. injection can be used without the reaction being too intense.

The method of Elliott was carried out as follows: Cats were anesthetized, a tracheotomy performed, both vagi cut, the brain destroyed, and the cord pithed to the midthoracic region. Artificial respiration was immediately started and continued throughout the experiment. The blood pressure was recorded from the common carotid, and the injections were made by means of a cannula in the femoral vein. An interval of twenty to thirty minutes was necessary after pithing the animal before the blood pressure falls to a constant level. Under such conditions the standard reaction may be obtained by 0.5 c.c. of adrenalin (Parke, Davis and Co.) in 1:100,000 dilution. The solutions are injected rapidly and followed by a little normal saline to rinse out the cannula. The reaction blood pressure curves may be compared and measured, giving the amount of adrenalin in the extracts compared with the standard solution, which can easily be estimated by the Folin method.

As stated above, some cats will not give a uniform reaction to pure adrenalin

or tissue extract given in equal or fractional repeated injections. These experiments must be rejected. A suitable interval between injections is imperative. Very often it will be found that the reaction to successive doses of pure adrenalin solution in equal amount will not be uniform. The curves will become higher until a maximum is reached when subsequent injections will give lower curves. It is important not to tax the system by overdoses of adrenalin, as the subsequent reactions to smaller doses will be much below the first reaction to such doses. It is unsafe to push the blood pressure much more than 60 mm. of mercury above the constant blood pressure level, as the cats may show breaks in the even rounded curve and subsequent reactions may be inaccurate. Our practice has been to make alternate injections of pure adrenalin solutions and unknown extract, using minimal amounts and increasing to an optimum reaction. The adjacent curves may then be compared and the amount of adrenalin computed.

EXPERIMENTAL OBSERVATIONS.

The following type experiment (table I) shows the effect of preservation and autolysis upon gland emulsions of normal pigs. The animals were killed by a blow on the head, bled at once, and dressed as usual. The gland hash was made at once and tested at intervals as indicated in the table. It is clear that the drop in the adrenal index is not very rapid during preservation on ice.

TABLE I.

Adrenalin Index. Chemical Method.

Normal fresh pig adrenal extract	1.604 mg. per gm.
Normal fresh pig adrenal extract plus 1 mg. adrenalin solution	2.540 mg. per gm.
Preservation on ice for 2 dys	1.578 mg. per gm.
Preservation on ice for 4 dys	1.463 mg. per gm.
Preservation on ice for 6 dys	1.200 mg. per gm.
Preservation[1] on ice for 8 dys	0.506 mg. per gm.

TABLE II.

Normal Dogs.

No. of animal.	Adrenalin in mg. per gm. of gland. Chemical method.	Physiological method.
94	1.396	...
107	1.360	...
12–105	1.416	4.31
N	1.83	5.70
12–140	1.167	3.09
0–92	1.25	4.55

Table II shows the normal adrenalin index for healthy dogs. These dogs were killed by means of ether anesthesia for about five

[1] At this time an odor of putrefaction was evident.

minutes and rapid bleeding from the carotid. The wide divergence of the chemical and physiological determinations will be discussed later (tables XIV to XVII).

TABLE III.

Fatal Shock. Dog Injected Intravenously with Poison Obtained from Closed Duodenal Loops.

No. of animal.	Adrenalin in mg. per gm. of gland. Chemical method.	Remarks.
0–30	0.450	Death in 4 hrs.
0–38	0.043	Death in 6+ hrs. Autopsy 12 hrs. post mortem.
0–44	0.388	Death in 4 hrs.
0–52	0.345	Death in 4 hrs.
0–53	0.303	Death in 4 hrs.
0–56	0.433	Death in 4 hrs. Autopsy 12 hrs. post mortem.
0–56	0.433	Death in 4 hrs. Autopsy 12 hrs. post mortem.
0–74	0.378	Death in 8 hrs. Autopsy 12 hrs. post mortem.
0–63	0.925	Death in 24 hrs. Bronchopneumonia.
110	0.417	Death in 2 hrs.

Table III shows the constant low adrenalin index of dogs poisoned fatally with fluid obtained from closed duodenal loops. The details of these experiments have been published elsewhere (Whipple, Stone, and Bernheim). The dogs die with constant symptoms of fatal shock, low blood pressure, and temperature, vomiting, and diarrhea, with profound collapse. The adrenalin index of dogs dying in four hours is about one fourth of normal, indicating perhaps a depletion of the adrenalin in an effort to compensate the lowered blood pressure.

TABLE IV.

Intoxication. Dog.

No. of animal.	Adrenalin in mg. per gm. of gland.		Remarks.
	Chemical method.	Physiological method.	
0–35	0.186		High intestinal obstruction. Death in 5 dys.
0–39	0.566		High intestinal obstruction. Death in 6 dys.
0–58	0.483		High intestinal obstruction. Death in 3 dys.
0–106	1.25	0.29	Closed duodenal loop. Killed on 3d dy.
0–100	1.083	1.02	Closed duodenal loop. Killed on 3d dy.
D–L	0.833	1.80	Closed duodenal loop. Death on 4th dy.
0–121	0.917	3.90	Closed duodenal loop. Killed on 3d dy.
0–66	0.511		General peritonitis. Loop rupture.
0–60	0.811		General peritonitis. Bile duct rupture.
0–68	0.566		Acute hemorrhagic pancreatitis. Bile injection.

Table IV confirms the experiments in table III and shows a constant low adrenalin index in the intoxication of intestinal obstruction and closed loop experiments. Acute pancreatitis and general peritonitis may give the same low index.

TABLE V.

Anesthesia and Liver Injury. Dog.

No. of animal.	Adrenalin in mg. per gm. of gland.		Remarks.
	Chemical method.	Physiological method.	
C–1	0.761		Killed at the end of 2 hrs. Chloroform anesthesia.
C–2	0.783		Perfusion for 3 hrs. Ether anesthesia.
0–117	1.00	3.35	Chloroform for 2 hrs. Killed 24 hrs. later.
12–63	1.125		Chloroform for 2 hrs. Killed 48 hrs. later.
12–59	0.532		Phosphorus fatal in 18 hrs.
12–76	0.975		Phosphorus fatal on 3d dy.
12–80	1.156		Phosphorus fatal on 6th dy.
12–64	0.018		Phosphorus subcutaneously. *B. coli* intravenously.
0–111	0.667	1.49	Liver extirpation. Killed in 5 hrs.
12–87	0.633		Hydrazine. Fatal on 2d dy.
12–93	0.811		Hydrazine. Fatal on 3d dy.

Table V shows the effects of chloroform or ether upon the adrenalin index. There is a fairly rapid return to normal. It is interesting that various liver poisons cause a definite fall in the adrenalin index. The more acute the injury the more pronounced is the diminution in adrenalin. The combination of a liver poison with a septicemia causes the most complete depletion of the adrenalin. This is true of chloroform as well as phosphorus, and such animals die in a few hours with symptoms of acute shock.

TABLE VI.

Pancreatic Diabetes. Cat.

No. of animal.	Adrenalin in mg. per gm. of gland. Chemical method.	Remarks.
1	1.26	Normal.
2	0.20	Death in 7 dys. Constant glycosuria.
3	0.27	Death in 8 dys. Constant glycosuria.

Table VI shows the effect of removal of the pancreas in cats. These two animals after operation showed constant glycosuria and

rapid loss of weight. This observation is of interest when compared with a human case of exophthalmic goitre (table VIII) with fatal intoxication showing a low adrenalin index; also compare with human diabetes with a low adrenalin index (table XI).

TABLE VII.

Miscellaneous Conditions. Man.

Case No.	Hrs. post mortem.	Adrenalin in mg. per gm. of gland.		Remarks.
		Chemical method.	Physiological method.	
3969	2	0.417	0.75	Cerebral hemorrhage. Blood pressure 140 mm.
3877	31	0.293	0.22	Cerebral hemorrhage. Blood pressure 170 mm.
3877		0.360	0.24	
3867	15	0.289		Ruptured aneurysm (thorax).
3867		0.286		
3878	3	0.493	0.40	Trauma (brain); bronchopneumonia.
3878		0.451	0.40	
3868	16	0.00		Tuberculous adrenal.
3868		0.733		Prostatitis and abscesses.
3916	4	0.867		Pernicious anemia.
3916		0.867		

Table VII brings out several interesting points. It may be assumed that the human cases that die suddenly from hemorrhage or trauma are approximately normal. These cases together with some others cited below indicate that human adrenals shortly after death in approximately normal adults show an adrenalin index of 0.35 to 0.50; that is, they contain 0.35 to 0.50 of a milligram of adrenalin per gram of gland, as estimated by the chemical method. Postmortem deterioration is probably not rapid when the bodies are kept in an ice box and there is no evidence of autolysis. Human glands (right and left) do not contain exactly the same amounts of adrenalin per gram of weight, but contain approximately equal amounts.

It is noteworthy that one case (No. 3868) with a caseous adrenal (adrenalin index 0) showed almost double the normal amount of adrenalin in the other gland which did not show marked hyperplasia.

The case of pernicious anemia is of interest because of recent investigations which indicate that the spleen and adrenal may be concerned in lipoid metabolism which is thought to be upset in this disease. Physiological overactivity shows an increase in the adrenalin index of the suprarenal. May we argue the converse, that a very high adrenalin index means an increased activity of the gland? If the gland cortex is overactive, it is possible that the medulla may be stimulated to a somewhat parallel overactivity. By contrast it is seen that secondary anemias show a low adrenalin index (tables X and XI).

TABLE VIII.

Intoxications. Man.

Case No.	Hrs. post mortem.	Adrenalin in mg. per gm. of gland.		Remarks.
		Chemical method.	Physiological method.	
3883	8	0.471		Eclampsia.
3883		0.327		
3965	24	——	0.111	Exophthalmic goitre, toxemia.
3881	24	0.157		Baby 16 mos. Rachitis.
3908	18	0.162		Baby 11 mos. Rachitis.
3908		0.161		

Table VIII shows a case of eclampsia with the characteristic liver lesion, which died with very acute symptoms of intoxication, to have a normal adrenalin index. Cases of severe rachitis show a low index. Exophthalmic goitre with acute intoxication shows a very low adrenalin index. It is unfortunate that a chemical determination was not done, but it is probable from experiments done at this time under similar conditions that the chemical test would have been lower than the physiological, perhaps one half or one third.

Table IX shows a considerable variety of acute infections. It is seen that some cases of general peritonitis, meningitis, and typhoid may show a normal adrenalin index. The average is somewhat subnormal, but the drop below normal is not marked, and we may conclude that acute infections may not cause a great depletion of adrenalin stored in the glands.

TABLE IX.

Acute Infections. Man.

Case No.	Hrs. post mortem.	Adrenalin in mg. per gm. of gland.		Remarks.
		Chemical method.	Physiological method.	
3905	16	0.353		Peritonitis, pericarditis, pleurisy, and bronchopneumonia.
3905		0.142		
3896	14	0.173		General peritonitis.
3896		0.175		
3863	9	0.473		General peritonitis.
3863		0.510		
3862	24	0.280		Baby 6 mos. Pneumonia, meningitis.
3866	4	0.245		Bronchopneumonia.
3866		0.205		
3872	7	0.184	0.44	Baby 16 mos. Bronchopneumonia.
3872		0.181	0.15	
3871	24	0.330		Meningitis, bronchopneumonia.
3871		0.286		
3875	4	0.415	1.00	Otitis media, meningitis.
3875		0.375	1.11	
3917	14	0.252	0.43	Endometritis, septicemia, icterus.
3917		0.231	0.32	
3939	4	0.157		Abortion, suppurative nephritis.
3899	6	0.182		Pylephlebitis, icterus.
3899		0.211		
3911	10	0.434		Typhoid fever, toxemia.
3911		0.625		

It may be noted that two glands (No. 3872) gave equal amounts of adrenalin by the chemical method, but three times the amount in one gland compared with the other by the physiological method. There was no anatomical difference in the two glands and we are inclined to believe that the chemical method was more nearly correct.

Table X shows that in acute tuberculosis the adrenalin index may fall to about one half normal. It will be noted that the glands on the two sides may vary considerably as to their adrenal content. The last case shows one half as much adrenalin per gram in one gland as in the other.

TABLE X.

Tuberculosis. Man.

Case No.	Hrs. post mortem.	Adrenalin in mg. per gm. of gland. Chemical method.	Remarks.
3891	15	0.220	Baby 22 mos. Miliary tuberculosis.
3891		0.249	
3898	16	0.250	Baby 7 mos. Miliary tuberculosis.
3898		0.330	
3924	15	0.058	Miliary and chronic tuberculosis.
3861	14	0.132	Pulmonary tuberculosis and ruptured aneurysm.
3861		0.287	

Table XI shows that the cachexia due to malignant tumors is associated with a low adrenalin index,—one half normal or even considerably less.

TABLE XI.

Neoplasms. Man.

Case No.	Hrs. post mortem.	Adrenalin in mg. per gm. of gland.		Remarks.
		Chemical method.	Physiological method.	
3903	1	0.205		Cancer of stomach, metastases.
3903		0.269		
3912	20	0.111		Cancer of esophagus, empyema.
3912		0.111		
3972	3	0.216	0.55	Cancer of prostate, suppurative nephritis.
3913	18	0.042		Cancer of stomach, general peritonitis.
3913		0.099		
3901	9	0.133		Hypernephroma, anemia.
3901		0.150		
3874	60	0.004	0.02	Adenoma of adrenal.
3874		0.012	0.20	Diabetes. Blood pressure 90 mm.

The case of malignant adenoma of the adrenal associated with diabetes is of especial interest. The diseased adrenal showed only a trace of adrenalin and the uninvolved gland showed a very low adrenalin index. We cannot attribute this solely to the post-mor-

tem change, as other autopsies of equal age show only a moderate fall below normal (tables XII and XIII). This observation harmonizes with those of table VI in which experimental pancreatic diabetes was associated with a low adrenalin index.

TABLE XII.

Nephritis. Man.

Case No.	Hrs. post mortem.	Adrenalin in mg. per gm. of gland.		Remarks.
		Chemical method.	Physiological method.	
3865	50	0.261		Chronic nephritis, large kidney.
3865		0.260		Blood pressure 200 mm.
3935	8	0.522		Chronic and acute nephritis.
3935		0.405		Bronchopneumonia, pericarditis. Blood pressure 165 mm.
3882	6	0.182	0.50	Chronic nephritis. Blood pressure 190 mm.
3882		0.240	0.40	Acute endocarditis.

TABLE XIII.

Cardiac Failure and Passive Congestion. Man.

Case No.	Hrs. post mortem.	Adrenalin in mg. per gm. of gland.		Remarks.
		Chemical method.	Physiological method.	
3970	56	0.162		Myocarditis, pericarditis.
3897	18	0.368		Myocarditis, anasarca.
3897		0.371		
3909	4	0.091		Myocarditis, thrombosis.
3909		0.087		
3973	8	0.333	0.48	Myocarditis, bronchopneumonia. Blood pressure 175.
3869	4	0.495		Mitral insufficiency, Pott's disease.
3869		0.450		

Tables XII and XIII show that increased vascular tension associated with renal, cardiac, or vascular disease does not cause any great fluctuation in the adrenalin index. If anything the values are somewhat subnormal, but this may be accounted for in some instances by the secondary infections.

METHOD CONTROL EXPERIMENTS.

Because of peculiar divergencies between the chemical and physiological determinations in some of the glands, group experiments were done in which as many unequal factors as possible might be eliminated. Our results do not agree with the series of observations of Folin, Cannon, and Denis in which the two methods agreed with startling uniformity. For this reason we were careful to eliminate as many sources of error as possible. In the following groups of experiments care was taken not to overtax the decerebrate organism by too great doses of adrenalin. The chemical determinations were done in duplicate and correspond roughly to the theoretical values. The physiological values at times are high and again low.

TABLE XIV.

No.	Adrenalin in mg. per gm. of gland.		Remarks.
	Chemical method.	Physiological method.	
12–140 (dog)	1.167	3.09–3.10	Normal.
0–92 (dog)	1.250	4.00–5.10	Normal.
0–117 (dog)	1.000	3.33–3.40	Chloroform anesthesia for 2 hrs. Killed 24 hrs. later.
0–119 (dog)	0.917	1.88–2.55	Tissue juices intravenously. Death in 10 hrs.
0–111 (dog)	0.667	1.26–1.73	Liver extirpation—ether. Killed at end of 5 hrs.
3972 (man)	0.216	0.43–0.64	Cancer of prostate. Suppurative nephritis.
3973 (man)	0.333	0.36–0.60	Myocarditis. Passive congestion. Bronchopneumonia. Blood pressure 175.

Table XIV shows a group of observations done at the same time under the same conditions, using the same reagents and decerebrate cat. The chemical adrenalin indices vary from the normal dog down through lower values in the dog due to anesthesia, to the human cases of low value. Two series of physiological tests were done and are arranged side by side in the same table. Some determinations correspond and others do not, showing a variation of almost 50 per cent. The physiological determination is much higher than the chemical but the ratio is not the same in the different glands.

TABLE XV.

Dog.

No. of animal.	Adrenalin in mg. per gm. of gland.		Remarks.
	Chemical method.	Physiological method.	
N	1.83	5.70	Normal.
12–41	2.17	6.60	Recovery in 3 wks. following chloroform poisoning.
0–112	2.17	6.30	Dog immunized to duodenal loop poison.
0–121	0.92	3.90	Closed duodenal loop. Killed on 3d dy.
0–115	1.25	4.00	Dog immunized to duodenal loop fluid.
D–L	0.83	1.80	Closed duodenal loop. Death on 4th dy.

Table XV also shows a group of observations done at the same time under identical conditions. Again the chemical values correspond relatively to the theoretical ratio, which might obtain between the individual cases. These are the highest physiological values which we have ever observed, and this decerebrate cat was very sensitive to minute doses of adrenalin. It is interesting to compare this table with table XVII, where the physiological is below the chemical adrenalin index, in one instance of one fourth value.

TABLE XVI.

No.	Adrenalin in mg. per gm. of gland.		Remarks.
	Chemical method.	Physiological method.	
12–105 (dog)	1.42	4.31	Normal.
0–109 (dog)	1.67	2.62	Duodenal loop poison (sublethal dose). Recovery in 36 hrs. Killed.
3969 (man)	0.42	0.75	Cerebral hemorrhage. Blood pressure 140 mm.

Table XVI shows another group of experiments done on the same day under identical conditions. The second experiment (dog 0–109) shows a higher adrenalin index by the chemical method, but by the physiological method about one half the normal control.

Table XVII shows two groups of experiments done on successive days under identical conditions. It will be seen that the physiological method gives an index below that given by the chemical method. The values by the chemical method are in harmony with other observations in similar experimental animals given above. This illus-

TABLE XVII.

Dog.

No. of animal.	Adrenalin in mg. per gm. of gland.		Remarks.
	Chemical method.	Physiological method.	
July 22			
0–106	1.25	0.29	Closed duodenal loop. Killed on 3d dy.
0–100	1.08	1.02	Closed duodenal loop. Killed on 3d dy.
0–102	0.75	0.63	Uremia(?).
July 23			
0–106	0.83		
0–100	0.67		Solutions kept for 24 hrs. in cool place, not on ice.
0–102	0.50		
0–110	0.42		Shock. Duodenal loop poison intravenously. Death in 2 hrs.
0–99	0.96		Immune dog with closed duodenal loop. Killed on 8th dy.

trates the wide fluctuations which may be observed in group experiments with the decerebrate cat. The last half of the table shows the rapid deterioration which may occur in the boiled filtered gland extracts kept in a cool dark place.

DISCUSSION.

The method of determining the adrenalin index, that is, milligrams of adrenalin per gram of gland, is open to error. In our hands the chemical colorimetric method has been much more uniform and subject to few sources of error. To the inexperienced worker the physiological method (with the decerebrate cat) is vastly more difficult than the chemical method. Different cats will not react in the same way, or they may react in the same way to pure adrenalin and in a different manner to the gland extracts. The tissue extract seems to affect different animals in a strikingly different manner. There are so many possibilities of individual variation among these cats as to cast doubt on any single series of observations with one animal. In our work if the cat had been given an overdose of adrenalin, perhaps causing only a rise of 60 to 80 millimeters of mercury, the subsequent reactions might be quite variable. The chemical method gives results which are more in harmony with the theoretical values, and known dilutions of a gland extract can be determined much more accurately than by the

physiological method. The same holds true for standardized gland extract, to a portion of which has been added a known amount of pure adrenalin. When the gland extract shows evidence of autolysis and a known amount of adrenalin has been added to a portion, it will be found that both methods may fail to estimate the adrenalin accurately. Tissue extracts in general tend to depress blood pressure, particularly products of autolysis, and this may explain the low results in some of the physiological determinations. It is possible that the adrenals contain some substance other than adrenalin which will cause elevation of blood pressure. It is difficult to explain some of the findings in the last few tables unless some such possibility is taken into account.

SUMMARY.

The adrenalin index as used in this paper means the amount of adrenalin in milligrams per gram of gland. As in our hands the chemical colorimetric method has proved more accurate, these values rather than the physiological values will be given in the final analysis. The two adrenal glands in the same individual as a rule contain about the same amount of adrenalin per gram, but variations of 10 to 20 per cent. are not unusual.

Normal dogs show an index which may vary from 1.2 to 1.8 milligrams. The dogs were killed by short ether anesthesia and bleeding from the carotid.

Normal human beings, dying from trauma, rupture of aneurysm, etc., show an index of 0.35 to 0.50 of a milligram, when autopsy takes place a few hours after death. Deterioration of uncut glands or of a gland hash kept on ice in the dark is not rapid and rarely exceeds 10 per cent. in twenty-four hours.

Acute intoxication in dogs shows a low adrenalin index, especially the intoxication associated with intestinal obstruction and the closed intestinal loop. Intravenous injection of the poison found in closed duodenal loops sufficient to cause fatal shock causes a great drop in the adrenalin index, at times to one fourth normal or even lower.

After recovery from a sublethal toxic dose the adrenalin index may rise rapidly to a point considerably above normal. The same may hold for recovery after chloroform poisoning.

Anesthesia by chloroform or ether causes a drop in the adrenalin index depending upon the length of anesthesia and probably in part on the depth of anesthesia.

Liver poisons (chloroform, phosphorus, hydrazine) cause a drop in the adrenal index to a low level, perhaps one half normal in acute cases.

Pancreas extirpation with prolonged glycosuria and death produces a great drop in the adrenalin index (cat). There is evidence that this may hold in some cases of human diabetes.

In man disease of one adrenal (tuberculosis) may be associated with an adrenalin index of double the normal value in the intact adrenal.

Pernicious anemia is the only disease so far found to present an abnormally high adrenalin index, and the single case shows an index at least twice normal. This is of interest especially in relation to the views recently put forward to indicate that the spleen and adrenal may be concerned in the lipoid metabolism which is thought to be profoundly disturbed in this disease.

Secondary anemia due to repeated hemorrhage or the intoxication of cancer or tuberculosis causes a fall in the adrenalin index. Cachexia due to neoplasm or tuberculosis may cause a marked fall in the adrenalin index, perhaps to less than one half of normal.

Acute infections (typhoid fever), septicemia, peritonitis, and similar conditions may be associated with a normal adrenalin index or one somewhat below normal.

Diseases of the kidneys, heart, or blood vessels associated with elevated blood pressure show no constant variation in the adrenalin index, which may be normal or slightly subnormal.

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OVARIAN INFECTION IN THE DOMESTIC FOWL AND DIRECT TRANSMISSION OF DISEASE TO THE OFFSPRING.*

By LEO F. RETTGER, Ph.D.

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In an extended discussion of the subject of inheritance and disease Adami[1] concludes that "specific infections are not inherited, but are of post-conceptional acquirement." Von Wassermann[2] holds the same opinion; in fact, this appears to be the generally accepted view. In the scientific interpretation of the term there can be no inheritance of disease. Parental properties are conveyed by the nuclear material of the spermatozoön and ovum; in other words, the inheritance of physiological and other properties is a function of the chromatin of the sperm and the egg. If germinal infection takes place and affects the offspring, the acquirement of the disease is by transmission only. Even this mode of handing transmission is disputed, however.

Instances of prenatal infection are too numerous to permit of doubt as to its occurrence, but it has at all times been difficult to exclude the possibility of intra-uterine infection, either as the result of a diseased condition of the placenta, or the passage of pathogenic organisms by way of the vagina through the amnion into the amniotic fluid. Syphilis is a common type of congenital disease, whereas tuberculosis appears to be transmitted rarely.

Calmette[3] claims that both placental and germinal transmission of tuberculosis are of uncommon occurrence in man. This view is strongly opposed by Baumgarten. In reviewing the literature Kerscher[4] found records of 181 alleged

* Received for publication, March 23, 1914.

[1] Adami, J. G., in Osler, W., and McCrae, T., A System of Medicine, London, 1907, i, 17.

[2] von Wassermann, A., and Keysser, Fr., in Kolle-Wassermann, Handbuch der pathogenen Mikroorganismen, 2d edition, Jena, 1912, i, 659.

[3] Calmette, A., *Ann. de l'Inst. Pasteur*, 1910, xxiv, 771.

[4] Kerscher, abstracted in *Jahresber. ü. d. Fortschr. in d. Lehre v. d. path. Mikroorgan.*, 1910, xxvi, 507.

cases of congenital tuberculosis in man. Of this number 55 appeared to him to be well authenticated. The instances of placental infection were estimated at 35. Landouzy[5] believes that the danger of congenital transmission from the mother is greater than from the father. Gaertner[6] cites a number of cases of congenital tuberculosis which are apparently of placental origin. Katholický[7] states that inherited tuberculosis is not rare.

Numerous instances of placental transmission of disease, besides syphilis, tuberculosis, and leprosy, are recorded;[8] for example, anthrax (Paltauf), pneumonia (Levy, Netter, Viti), typhoid fever (Eberth, Ernst, Freund, Levy), pyogenic coccus infection (Auche, Lebedeff, Fraenkel), recurring fever (Spitz), and variola (Champ). Scarlet fever, measles, malaria, articular rheumatism, glanders, and Asiatic cholera are claimed to have been transmitted in this way, also.

As to germinal infection, particularly in man, there has been less conclusive evidence. Baumgarten has for many years held the view that germinal transmission plays an important part in congenital tuberculosis. His views have been accepted by other investigators, but have not been substantiated by scientific observation. The instances of apparent ovarian infection in tuberculous women, and in guinea pigs that were experimentally infected with tubercle bacilli, have been too few and too uncertain to allow positive conclusions to be drawn from them.

Rosinski[9] strongly adheres to the theory of germinal transmission of syphilis, and claims to have demonstrated the certainty of paternal transmission through the spermatozoa. In as far as ovular infection is concerned, the egg may become infected, at the time of its development and great physiological activity, by organisms which may have been latent for some time. Neumann[10] also accepts the idea of ovarian transmission of syphilis to the offspring, and believes it is more common than the placental. The unprotected ovum appears to him to be more liable to infection than the fetus which is enclosed in the placenta. He furthermore demonstrates paternal transmission of disease through the sperm cells.

Sibley[11] reported an incident which has considerable bearing on the question of germinal infection. Eggs for hatching purposes were exchanged between two farmers. One of the farms had apparently always been free from avian tuberculosis. Soon after the introduction of the new stock (exchanged eggs) tuberculosis appeared in acute form.

Maffucci[12] inoculated eighteen eggs with a broth culture of the avian tubercle bacillus. After natural incubation of these eggs eight chicks were obtained.

[5] Landouzy, abstracted in *Jahresber. ü. d. Fortschr. in d. Lehre v. d. path. Mikroorgan.*, 1910, xxvi, 508.

[6] Gaertner, *Ztschr. f. Hyg. u. Infectionskrankh.*, 1893, xiii, 131.

[7] Katholický, abstracted in *Jahresber. ü. d. Fortschr. in d. Lehre v. d. path. Mikroorgan.*, 1903, xix, 476.

[8] Cited by von Wassermann, A., and Keysser, Fr., *loc. cit.*

[9] Rosinski, abstracted in *Jahresber. ü. d. Fortschr. in d. Lehre v. d. path. Mikroorgan.*, 1904, xx, 690.

[10] Neumann, *Wien. klin. Wchnschr.*, 1904, xvii, 551.

[11] Sibley, cited by Gaertner, *loc. cit.*

[12] Maffucci, A., *Centralbl. f. Bakteriol.*, 1889, v, 237.

All but one of the chicks were active, but small and delicate; one died thirty-six hours after hatching, without indications of tubercular infection. The others died after periods varying from twenty days to four and a half months. At least six out of the eight chicks showed marks of tubercular infection, and in several of these the presence of tubercle bacilli was demonstrated. Baumgarten[13] inoculated twelve eggs with tubercle bacilli. Only two chicks were obtained from these eggs, but both gave evidence of tuberculosis.

Gaertner[14] injected tubercle bacilli of human origin into the peritoneal cavity of twelve female canary birds. All succumbed to tuberculosis. The contents of two of the nine eggs laid by these birds produced tuberculosis in guinea pigs, when injected into the peritoneal cavity. The remaining seven eggs gave apparently negative results.

These experiments strongly indicate that germinal infection and transmission can, and undoubtedly do, take place, at least in fowls. They are fully supported by the observations upon which this paper is based.

The investigations of Pasteur and others on pébrine have shown conclusively that infected silkworm eggs are a grave source of danger to the propagation of the silkworm, and that the selection of sound and uninfected eggs is necessary for the success of this industry. Little, if anything, seems to be known as to the manner in which the eggs become infected; whether in the process of formation, during the act of expulsion, or as the result of contact with infected matter after the eggs are laid.

There is also abundant evidence that the organism of Texas or Southern cattle fever, the piroplasma of Smith, is carried in the eggs of the tick (*Boöphilus bovis*). Here again the mode of infection of the eggs has not been determined, but that eggs from ticks which harbor the specific piroplasma are important agents in the transmission of this cattle disease has been satisfactorily shown.[15]

EXPERIMENTAL.

The following report is based entirely on a series of extensive observations made by the writer and his associates upon ovarian infection in the domestic fowl. This study was part of a general investigation of the economically important disease known in all parts of this country as bacillary white diarrhea of young chicks.[16]

Bacillary white diarrhea of chicks is a poultry scourge which was practically unknown prior to 1900. In its common manifestations it affects only chicks which are less than five or six weeks old. The greatest mortality occurs during the first two weeks. The symp-

[13] Baumgarten, cited by von Wassermann, A., and Keysser, Fr., *loc. cit.*

[14] Gaertner, *loc. cit.*

[15] Smith, T., and Kilborne, F. L., *Annual Reports of the U. S. Bureau of Animal Industry*, 1891–92, Nos. 8 and 9, 45, 177.

[16] Rettger, L. F., and Stoneburn, F. H., *Bull. Storrs Agricultural Experiment Station*, December, 1909, No. 60, 33; April, 1911, No. 68, 279. Rettger, L. F., Kirkpatrick, W. F., and Stoneburn, F. H., *idem*, December, 1912, No. 74, 153.

toms are those of acute bowel trouble; namely, diarrhea, listlessness, loss of appetite, subnormal temperature, and extreme weakness, especially in the later stages.

One of the earliest known epidemics came to the writer's notice in the summer of 1899. During an investigation of this epidemic an organism was isolated from the liver, heart, and lungs of chicks which died from the disease. Inoculation of normal chicks with this organism resulted in the death of the chicks, the symptoms and post-mortem appearances being the same as those for typical bacillary white diarrhea. In the numerous epidemics which have been studied since that time the same organism has invariably been found. Its definite relation to the disease has been established repeatedly by inoculation and artificial feeding experiments. A brief description of this microörganism, now generally known as *Bacterium pullorum,* follows.

Morphology, Cultural Characteristics, etc., of Bacterium pullorum.—The organism is a long, slender bacillus (0.4 to 0.5 × 2 to 4 micra) with slightly rounded ends. It usually occurs single, chains of more than two bacilli being rarely found. It is a non-motile, non-liquefying, non-chromogenic, facultative anaerobe. It is stained readily by the ordinary basic anilin dyes, but does not retain its stain by the Gram method. It does not produce spores.

Agar Plates.—Small colorless colonies make their appearance in twenty-four hours at 37° C. They increase in size slowly, and seldom attain more than one millimeter in diameter. They may be round, oval, or spindle-shaped. The surface is marked with one or two rosette figures. The border is comparatively smooth.

Slant Agar.—When streaked in the usual manner, a visible growth is obtained in twenty-four hours which spreads little, and remains delicate, even after three or four days of incubation. When, however, the entire surface of the agar is streaked with a platinum loop the characteristic cultural appearance of the pyogenic streptococcus is obtained, the growth being made up of minute discrete colonies which may be so small as to require a magnifying lens for their detection. This cultural characteristic is of extreme importance in identification work.

Bouillon.—The growth is like that of *B. typhi,* though as a rule not so luxuriant.

Gas Production in Sugar Bouillon.—Some of the strains produce gas in dextrose and mannite broth, whereas others lack this property. No reactions of any importance are obtained in any of the other laboratory sugar media.

Indol and Nitrite Production.—Neither indol nor nitrite could be detected in Dunham's peptone solution at the end of one week's incubation.

In its morphological, staining, and in certain of its cultural characteristics *B. pullorum* resembles *B. typhi*, and must, therefore, be classed as a member of the coli-typhi-enteritidis group of bacilli.[17]

Numerous feeding experiments in which the food was artificially infected with *Bacterium pullorum* demonstrated without any doubt that bacillary white diarrhea is highly infectious, and that it is transmitted from chick to chick through infected droppings. This fact furnishes an explanation of the rapid spread of the disease and its frequent occurrence in epidemic form. It does not, however, establish the immediate source of infection in those chicks which are the first to become affected.

THE OVARIES OF THE BREEDING STOCK AS THE IMMEDIATE SOURCE OF INFECTION.

One of the peculiar conditions that had frequently been observed in diseased chicks was the failure to absorb the yolk. In fact, this is one of the most striking symptoms of the disease, and makes itself manifest in the protrusion of the abdomen below the vent. Bacteriological examinations of the contents of the yolk sac invariably revealed the presence of *Bacterium pullorum* in the yolk.

It was but natural to assume that the yolk played an important part in bringing about a diseased condition of the chick while still in the egg. This assumption was further supported by the successful search for the bacterium in question in the yolks of infected chicks at the time of hatching, and of chicks at various stages of their development within the egg. Furthermore, when eggs which came from breeds of hens whose progeny had been known to be subject to white diarrhea were examined, *Bacterium pullorum* was frequently found. Not only eggs which were in various stages of incubation harbored the organism in the yolk, but perfectly fresh eggs as well.

It required but one more link in the chain of evidence to trace white diarrhea infection to the laying hen; namely, the demonstration of the presence of the organism in the ovary. This was ac-

[17] For a more detailed account of *B. pullorum*, see Rettger, L. F., and Harvey, S. C., *Jour. Med. Research*, 1908, xviii, 277; 1909, xxi, 115. Rettger, L. F., and Stoneburn, F. H., *Bull. Storrs Agricultural Experiment Station*, December, 1909, No. 60, 33.

complished without difficulty. Small flocks of hens, the eggs and chicks of which had revealed a history of white diarrhea infection, were killed and examined. Out of the first lot of 23 hens, 21 had abnormal ovaries which were found to contain *Bacterium pullorum* unaccompanied by any other organism.

A normal ovary which is undergoing natural development is made up of numerous ova of varying sizes. Some of the ova may be as large as an egg yolk; on the other hand, there are numerous minute ova many of which require magnification in order to be seen. The small ova are round and colorless, and may be likened to small tapioca grains that have been soaked in water. The color of the developing ova varies from a light yellow to a rich normal yolk color.

A typical infected ovary is composed of two distinct types of ova, the normal and the abnormal. The normal resemble those of perfect ovaries. The abnormal may vary as to size, and occasionally as many as twelve or more comparatively large ova, of practically uniform size, may be seen. The small cysts are usually less angular and irregular than the large ones. The color of the large cysts may vary greatly. Some are light, others dark; almost all shades of yellow and brown may be present. Occasionally the color suggests gangrene. The larger abnormal ova are usually quite angular in form, and of a firm consistency. At times they are so compressed as to appear flattened. The contents of the pathological ova are quite characteristic, consisting chiefly of a solid cheese-like matter which is permeated by a clear amber-colored fluid.

An infected ovary can be recognized readily, even by those who have had no special training. Hence, the direct examination of ovaries constitutes one of the best methods of determining the presence of white diarrhea infection in breeding stock. The bacteriological examination of the pathological ova is desirable, however, as a corroborative test.

CYCLE OF INFECTION.

Three possible explanations as to the manner in which ovarian infection with *Bacterium pullorum* is brought about naturally present themselves. First, chicks which have been victims of, but which have survived an attack of bacillary white diarrhea, may continue to harbor the organism of the disease for long periods of time, even to maturity, when the infection becomes localized in the ovary; second, fowls may become diseased by the ingestion of food and water which have been contaminated by the droppings of infected chicks, or carriers; and third, infection may be brought about by

association or contact with fowls which harbor the organism in question, as through the agency of body lice or of mites. There is also a possibility that the male may play some part in the transmission of the bacterium, although numerous attempts to demonstrate this have failed, and no evidence can be gathered that *Bacterium pullorum* is ever present in the testes.

It has been definitely shown that mature hens may acquire infection by feeding on food that has been artificially infected with broth cultures of *Bacterium pullorum,* and that the ovaries in particular become involved; but there is much doubt as to the probability that fowls which contain the organism in the ovum as the localized seat of injury eliminate the organism in the droppings, so that their excreta are a menace to other fowls. Although the question as to whether ovarian transmission from adult to adult hens takes place to an appreciable extent needs further elucidation, the carrying of infection to full maturity by chicks that survive the disease has been conclusively demonstrated in our investigations of the past year.

The experiments were conducted as follows.

A large number of chicks which were from twenty-four to forty-eight hours old were employed. They were divided into two lots, one of which was artificially infected with broth cultures of *B. pullorum,* while the other was not given this treatment, but served as a control for the first lot. In each experiment typical white diarrhea made itself felt throughout the artificially infected pens, with a high mortality, whereas the control pens remained unaffected. After the disease had fully run its course, or when the chicks were from seven to eight weeks old, the female chicks were transferred to permanent poultry houses where they were kept under daily observation until they were a year old, when the final examinations were made. The houses and the yards into which they opened had been thoroughly cleaned and disinfected, and had been left unoccupied for at least three or four weeks.

Of the 138 chicks which grew to maturity and lived until the termination of the experiments, 88 were known to have been infected with the organism in question, while the remaining 57 were survivors of the original controls. Of the 88 that were infected as chicks, 21 gave positive agglutination tests when they were about a year old, and showed unmistakable evidence of ovarian infection with *B. pullorum* at the time of autopsy. In other words, almost 25 per cent. of the chicks which were known to have been infected became bacillus carriers, the ovaries being apparently the only organs that were involved.

On the other hand, but one of the control lots showed any indications of ovarian infection, 56 of the 57 controls giving negative results in the agglutination tests and at the final examination. It is possible that the single exception may have suffered accidental infection as a chick or later in life.

Since the female chicks had been separated from the males at an early age (seven to eight weeks), and as no males were kept in the permanent houses, it may be concluded that the ovarian infection was brought about as the result of early infection of the chicks; hence our evidence as to a cycle of infection in bacillary white diarrhea is complete. The original source of infection is the ovary of the mother hen. The infected ovary produces eggs which carry the bacterium of white diarrhea in them; consequently chicks which are developed in these eggs are infected at the time of hatching. The infected chicks become an immediate source of danger to other or normal chicks, and epidemics of white diarrhea take place. Surviving female chicks become permanent bacillus carriers, and when mature they harbor the organism of the disease permanently in the ovary.

Numerous ovaries that have been examined by us have been so badly diseased in the manner described that the production of normal egg yolks had probably long ceased. On the other hand, there is abundant evidence that fowls which possess pathological ova continue laying through an entire laying season or longer. It is probable, too, that the most active layers are the most susceptible to ovarian infection, since the physiological activity of the ovary is such as to lessen its vitality and make it an easy prey to an organism which attacks the ovary more easily than any other organ. On the other hand, an ovary which is comparatively inactive or dormant is less apt to be disturbed.

As a rule, only a comparatively small number of the eggs that are laid by fowls having abnormal ovaries contain the organism of bacillary white diarrhea. It is often necessary to examine as many as eight or ten eggs from a single fowl before any positive evidence may be obtained by this method as to ovarian infection, although the ovaries are decidedly involved. This fact greatly lessens the value of routine egg-testing as a diagnostic measure, especially in as far as selection of individual fowls for breeding is concerned. As an aid in determining ovarian infection in flocks of fowl, however, it is of much importance.

The comparative infrequency with which *Bacterium pullorum* is, as a rule, transmitted to the egg, even when the ovary has been gen-

erally affected by the organism, is but another illustration of the interesting fact that nature does her utmost to protect the offspring in its period of embryonic development.

It is also of particular interest to note that eggs which carry the bacterium in question in their yolks harbor the organism in such small numbers that it is practically impossible to detect it unless the entire yolk is employed in the examination, or unless the eggs have been incubated at body temperature for at least three or four days, or until multiplication of the organism within the yolk has taken place. It is certain that while the yolk is held within the ovary or the oviduct of the fowl there is little or no reproduction of *Bacterium pullorum;* it is only after the time of laying that the apparently inhibitive influence of the yolk on the bacterium is reduced or completely lost.

There is no apparent difference between infected and uninfected eggs, aside from the presence of *Bacterium pullorum* in the former, and this can be detected by cultural methods only. In shape, size, color, odor, taste, and consistency the yolks of eggs that contain the bacterium of bacillary white diarrhea can not be distinguished from those of normal eggs.

The contents of eggs that are produced by normal fowls are, with rare exceptions, sterile at the time of laying and for several weeks after, if the shells remain whole, dry, and clean. This has been conclusively demonstrated by the writer.[18] Furthermore, numerous bacteriological examinations have shown that ovaries which are not infected with *Bacterium pullorum,* and which appear to be normal, are invariably sterile. The oviduct has also been found to be sterile to within a distance of three or four inches of the cloaca. It may be said, therefore, that in the fowl nature ordinarily makes the necessary provisions for the safeguarding of the young against early invasion by microörganisms.

The claim is not made here that the demonstration of ovarian infection with *Bacterium pullorum* in fowls is proof that germinal transmission of disease in mammals, and especially in man, can or does take place; but it makes the possibility of such transmission in man all the more apparent. The mere fact that the ova of fowls

[18] Rettger, L. F., *Centralbl. f. Bakteriol., 2te Abt.,* 1914, xxxix, 611.

and mammals are different as to their structure and development is not sufficient evidence that bacterial invasion of the mammalian ovule is impossible or even improbable. The yolk or food substance in the egg of fowls is not necessary for the successful invasion and establishment of *Bacterium pullorum,* as all the evidence at hand indicates that this organism does not multiply, at least to any noticeable extent, in the ovum or yolk until long after the egg leaves the body, but that it lies dormant during the entire period of ovular development, and even for a time after the egg is completed. These statements apply only to ova that develop normally and are expelled into the oviducts, and not to the pathological cysts that are retained in the ovary.

These observations are in accord with Baumgarten's view regarding germinal transmission as well as with the opinions of other investigators to whom reference has already been made.

SUMMARY.

Ovarian infection and germinal transmission of disease have been conclusively demonstrated in our investigations of bacillary white diarrhea in the common domestic fowl. The disease, which has caused so much loss to the poultry industry in recent years, primarily affects young chicks that are but a few weeks old.

Chicks which survive frequently become permanent bacillus carriers, the ovary being the important seat of infection. The eggs from such carriers often harbor the organism of the disease in the yolk. Chicks that develop in infected eggs become in turn infected, and have the disease at the time of hatching. The disease is transmitted to normal chicks through the infected droppings; thus an epidemic is produced, and the cycle of infection is completed.

There is no evidence to indicate that germinal transmission through the male takes place. In view of the frequent negative results bearing on this question it seems probable that it does not.

MORPHOLOGICAL AND DEVELOPMENTAL ANOMALIES OF A PATHOGENIC STRAIN OF TRYPANOSOMA LEWISI AND THEIR RELATION TO ITS VIRULENCE.*

By WADE H. BROWN, M.D.

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Plates 67 and 68.

The occasional references to unusual manifestations of virulence and the numerous descriptions of morphological and developmental anomalies of *Trypanosoma lewisi* contain scarcely a suggestion of a connection existing between changes in the virulence and changes in the morphology and developmental tendencies of the organism. The first suggestion of the possibility of such a relation is contained in the work of Wendelstadt and Fellmer,[1] on the effect of the passage of *Trypanosoma lewisi* through cold-blooded animals. These authors noted an increase in the virulence of the trypanosome for rats, and, at the same time, a profound alteration in the morphology of the organism, the main feature of which was a marked prolongation of the posterior extremity.

In a recent article[2] I published an account of an unusual increase in the virulence of a pathogenic strain of *Trypanosoma lewisi.* As this strain showed many morphological and developmental peculiarities, the life cycle and morphology of the organism were studied in eighty-two rats, with especial reference to the nature of the infection. In different series of infections, the rate of passage and the dose of trypanosomes were varied in an attempt to modify the resulting infections. As a control to this series, a study was made of a parallel series of rats infected with a typical benign strain of

* Received for publication, April 13, 1914.

[1] Wendelstadt, H., and Fellmer, T., *Ztschr. f. Immunitätsforsch., Orig.,* 1909, iii, 422; 1910, v, 337.

[2] Brown, W. H., *Jour. Exper. Med.,* 1914, xix, 406.

Trypanosoma lewisi that showed no morphological or developmental peculiarities. The details of these experiments and the effect of such procedures, as indicated, upon the course of the infection will be presented in another paper.

The usual life cycle and morphology of *Trypanosoma lewisi* in the rat's blood are so well known that they require no description, and it is not my purpose to present an exhaustive study of the anomalies of this organism and the factors giving rise to them, but rather the relation of anomalous development and morphology to the virulence exhibited by this particular strain. Therefore, only those deviations from the normal will be described that were pronounced and occurred with sufficient frequence in both fresh and stained preparations to be regarded as characteristic of the strain or of a particular infection. Most, if not all, of the peculiarities of this strain have been recognized by other workers but some of them are comparatively rare and apparently have not all been recognized in a single strain, while the conditions giving rise to these anomalies are but little understood.

DEVELOPMENTAL ANOMALIES.

In addition to the usual forms of multiplication, two clearly differentiated types of longitudinal division were observed; both of these were examples of equal binary fission; in rare instances the division was unequal or was multiple. The first of these types of division (figures 1 and 2) is more closely related to the usual process of multiplication as it occurred in trypanosomes showing the increased size, granular and basophilic cytoplasm characteristic of this period in the life cycle of the organism. Such forms of division were never numerous and were observed only during the usual period of multiplication. The sequence in the process of division could not be determined with certainty, as our only means of recognizing these forms comes with the division of the flagellum and undulating membrane. It is certain that division of these structures may occur first, but it is impossible to say whether trypanosomes with a divided nucleus or blepharoplast may not ultimately divide in this manner.

The second type of longitudinal division (figures 3 to 7) occurred far more frequently than the first and was observed during all phases of the blood infection. These forms presented a great variety of sizes, occurring in organisms that were quite large as well as in exceedingly small ones, but the general contour, nature of the cytoplasm, and staining reactions of all were characteristic of adult trypanosomes. A study of a large series of these forms indicated that the usual sequence of division was flagellum, undulating membrane, nucleus, and blepharoplast; but again, the sequence was undoubtedly subject to variation.

Several examples of multiple longitudinal division of adult trypanosomes were seen, one of which is shown in figure 7, but unequal division of this type of organism was not observed.

From published accounts of longitudinal division of *Trypanosoma lewisi,* it is uncertain whether both of these types of division have been recognized; the second is undoubtedly the one that is generally described. In a sense, the two types might be regarded as but one, since they grade into each other and probably represent a common tendency initiated in the young trypanosome and persisting in the adult organism even after all other forms of multiplication have disappeared from the blood.

The almost endless variety of forms exhibited by *Trypanosoma lewisi* in the usual process of multiplication precludes any consideration of the morphology of such organisms except as regards the degree of irregularity. Further, during the period of multiplication variations in the rate, extent, and persistence of multiplication may occur which markedly alter the character of the infection. At least two types of deviation from the normal have been recognized as indicating an increased severity of infection; either multiplication may be very rapid and marked with great numbers of irregular forms, especially small and imperfectly developed trypanosomes, or, with a normal rate and type of multiplication, this period of the life cycle does not cease at the usual time but continues on through the second or even the third week of the infection. According to our experience, the first type of infection is typically acute and occasionally terminates fatally at the height of multiplication, while the second, although usually more severe, progresses more slowly, and

fatal terminations occur, as a rule, after the second week of the infection.

ATYPICAL MORPHOLOGY.

Adult forms of *Trypanosoma lewisi* are generally uniform in character. The variations that we noted concerned the size, the nature of the flagellum and undulating membrane, the character of the posterior extremity, and the nucleus and the blepharoplast of the trypanosome.

Extremely small trypanosomes, measuring no more than 7 to 8 micra from the tip of the posterior extremity to the origin of the free flagellum, but possessing all the other characteristics of the normal adult trypanosome, were numerous in some severe infections. These small trypanosomes (figures 8 and 9) are of especial interest, as they appear to be identical with the small forms regarded by Swellengrebel and Strickland[3] as peculiar to the life cycle of *Trypanosoma lewisi* in the invertebrate host.

The posterior elongated forms of *Trypanosoma lewisi* described by many authors, and erroneously regarded by some as "of constant occurrence and very numerous at a certain stage of the multiplication-period,"[4] were very numerous in this strain of the organism. In some instances 10 per cent. of the trypanosomes showed this peculiarity (figure 10). Two types of elongated extremity were observed with about equal frequency. One of these possessed a delicately pointed tip (figures 11 and 12), and the other a bulbous extremity (figures 3 and 13). These posterior elongated forms were observed during all periods of the infection, but were most numerous during the latter part and immediately following the multiplication period. While this variety of trypanosome was usually larger than the normal adult, extremely small and irregular forms, such as those in figures 14 and 15, were occasionally seen. Other peculiarities of structure that usually accompanied an elongation of the posterior extremity were a highly developed and plicated undulating membrane and a short free flagellum, both of which are shown typically in figure 11. In a few fortunately stained prepa-

[3] Strickland, C., and Swellengrebel, N. H., *Parasitol.*, 1910, iii, 436.

[4] Minchin, E. A., An Introduction to the Study of the Protozoa, London, 1912, 292.

rations of these organisms a series of extremely faint longitudinal striations were seen that suggested the presence of myonemes.

Trypanosomes with an abnormally short posterior extremity (figure 16) were not infrequently observed along with the elongated variety. Occasionally there was a virtual absence of the postblepharoplastic segment, the blepharoplast being practically at the tip of the extremity.

The undulating membrane and the flagellum of *Trypanosoma lewisi* may vary quite independently of the posterior extremity. The two trypanosomes in figure 17 show extremes in the development of the undulating membrane, while figure 18 shows an organism in an early stage of division with an unusually prominent membrane. Figure 19 illustrates an extreme shortening of the free flagellum in an otherwise peculiar trypanosome.

Innumerable irregularities of the nucleus and blepharoplast have been described in detail by various authors and most of them are properly regarded as involution or degeneration phenomena. I observed total absence of a stainable nucleus, resulting from atypical division, in a few instances. This anomaly occurred in young trypanosomes with no evidence of degeneration, as well as in adult organisms where degeneration could not be excluded.

The production of ablepharoplastic trypanosomes through the agency of drugs has attracted so much attention that the spontaneous occurrence of this anomaly in *Trypanosoma lewisi* deserves especial mention. Figures 20 to 22 show ablepharoplastic trypanosomes that are either young or early multiplication forms with no evidence of degeneration or mechanical distortion. Absence of a blepharoplast was noted in all types of *Trypanosoma lewisi,* but was most frequent in the adult organism. While in most instances there was not the slightest suggestion of a blepharoplast, occasionally there was an extremely small granule (figure 20), representing a rudimentary blepharoplast. Although this type of organism was constant, and even numerous in many infections, it could not be found in other infections of the same strain of *Trypanosoma lewisi.* Examples of the developmental and morphological anomalies of this strain might be greatly extended, but these are sufficient to show the unusual character of the strain.

RELATION OF DEVELOPMENTAL AND MORPHOLOGICAL ANOMALIES TO VIRULENCE.

In studying these features of the organism with reference to the character of the infection, as indicated earlier in this paper, certain facts were brought out that indicated a degree of correspondence between the occurrence of developmental and morphological anomalies and the virulence of the strains with which I was working. As the blood of each of the eighty-two rats infected with our pathogenic strain of *Trypanosoma lewisi* showed most of the anomalies of development and morphology that have been described, these features may be regarded as characteristic of the strain. The control strain which, when first isolated, produced very benign infections and showed no anomalies, subsequently showed an increased virulence in certain series of infections coincident with the appearance of anomalous forms of trypanosomes in the blood of infected rats. The simultaneous alteration of these several properties of the organism suggests something more definite than a mere coincidence.

Further, in both strains the relative numbers and the variety of atypical trypanosomes differed in different infections and were greatest in two classes of infections, both of which have already been described as atypical infections. In one of these the incubation period was short, multiplication was rapid and pronounced, and there were enormous numbers of trypanosomes in the peripheral circulation. Some of these infections were unusually severe and occasionally terminated fatally, while others, in which multiplication ceased early and abruptly, showed a very mild course throughout.

The second class of infections in which anomalies were prominent differed from the first in that the evolution of the infection was more gradual and the period of multiplication persistent. The prominent features of these infections were the persistence of multiplication and the severity of the infection.

In conclusion, then, while it was certain that an intimate relation existed between the developmental tendencies and the morphology of these strains of *Trypanosoma lewisi,* it could not be definitely determined to what degree these characteristics were coördinated with the virulence. There were some facts that might lead one to believe

that as virulence influenced the course of the infections it also exercised an influence upon the developmental tendencies and hence the morphology of the organisms. My observations, however, inclined me to the opinion that, in so far as *Trypanosoma lewisi* was concerned, developmental tendencies exercised the dominant influence upon the morphology of the organism, and, while these characteristics did not correspond rigidly with the virulence of the organism, that anomalies of development and morphology, to some degree, corresponded with the virulence as manifested in the infections of *Trypanosoma lewisi* that I studied.

SUMMARY.

1. The morphological and developmental anomalies of a pathogenic strain of *Trypanosoma lewisi* have been described to show the unusual character of the strain.

2. Especial attention is called to the spontaneous occurrence of ablepharoplastic forms of *Trypanosoma lewisi.*

3. It is pointed out that morphological anomalies were most pronounced in infections that showed unusual conditions of multiplication, and that such infections usually proved severe.

4. Finally, an appreciable correlation between the morphological and developmental characteristics and the virulence as manifested in these examples of infection with *Trypanosoma lewisi* has been suggested.

EXPLANATION OF PLATES.

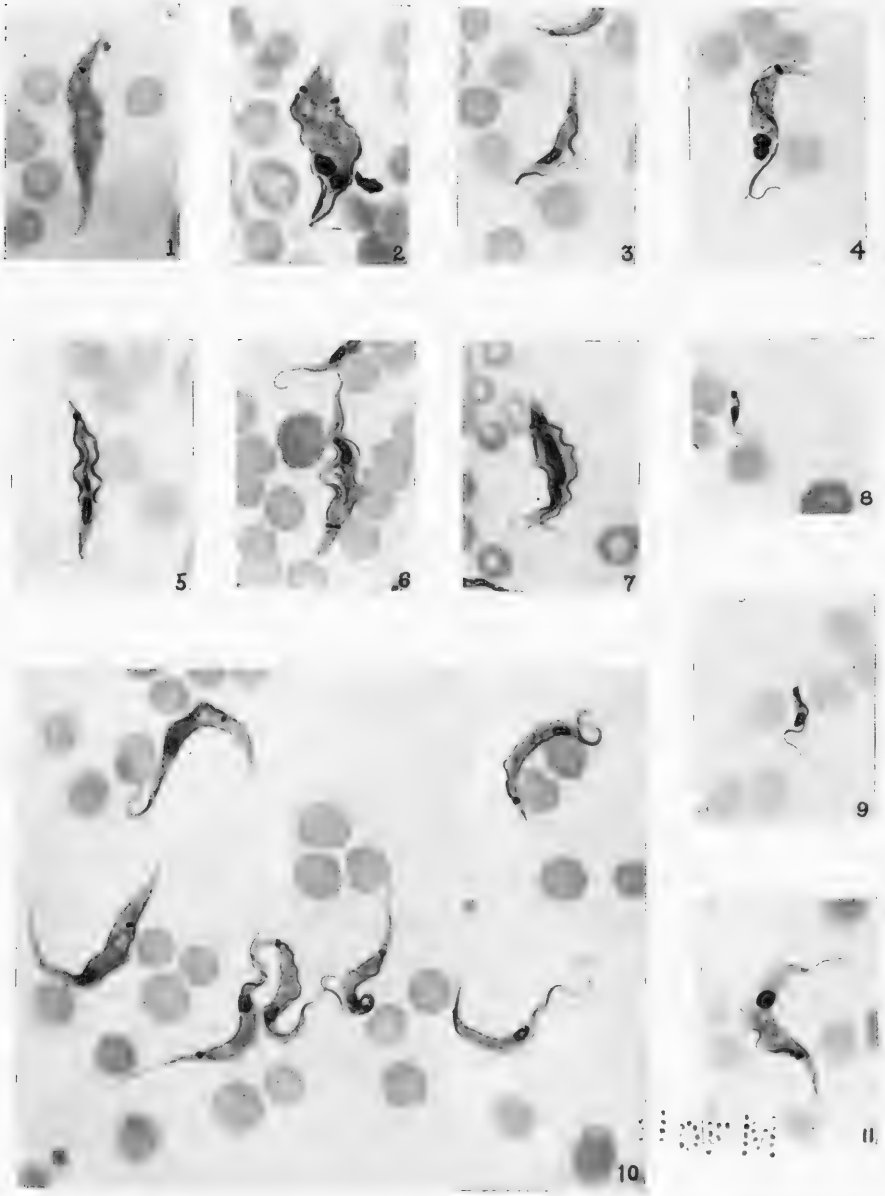
PLATE 67.

The photomicrographs show a magnification of 1,000 diameters, except figure 10, in which the magnification is 1,050. Figures 1, 3, 5, 7, 10, 16, 18, 21, and 22 are from smears of rat blood stained with Wright's blood stain. All the other figures are from similar preparations stained with Giemsa stain. The figures are not retouched.

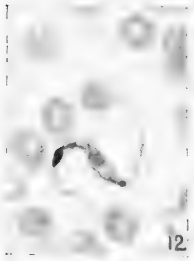
FIGS. 1 to 7. Longitudinal division of *Trypanosoma lewisi.* Figures 1 and 2 show trypanosomes with the usual cytoplasmic characteristics of organisms in process of multiplication, while figures 3 to 7 represent division of older forms. Figure 3 shows division of the flagellum in a trypanosome with an elongated posterior extremity, and figure 7 multiple division of an adult trypanosome.

FIGS. 8 and 9. Small trypanosomes with the characteristics of the adult organism.

FIGS. 10 to 15. Trypanosomes with an elongated posterior extremity.



(Brown: Anomalies of Pathogenic Strain of *Trypanosoma lewisi*.)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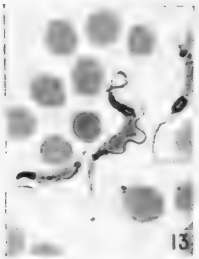
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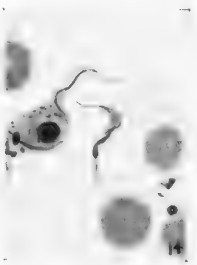
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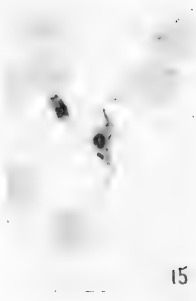
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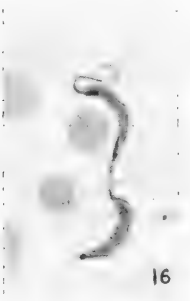
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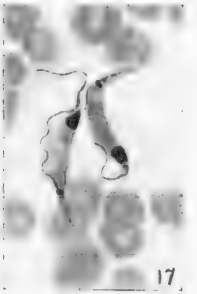
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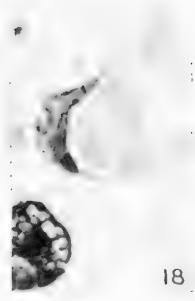
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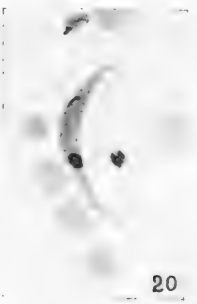
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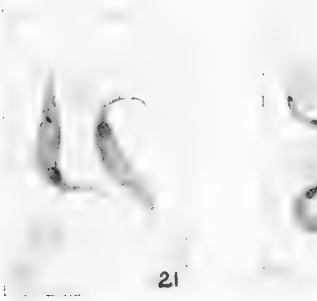
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(Brown: Anomalies of Pathogenic Strain of *Trypanosoma lewisi*.)

FIG. 10. Three adult trypanosomes with posterior elongations. Compare these with the two young forms, also with elongated posterior extremities, and with the normal adult trypanosomes.

FIG. 11. A very large trypanosome with a delicately pointed posterior extremity and a highly developed undulating membrane.

PLATE 68.

FIG. 12. An extreme elongation of the posterior extremity.

FIG. 13. Posterior extremity with a bulbous tip. Compare with figure 3.

FIG. 14. Small anuclear trypanosome.

FIG. 15. A young trypanosome with posterior elongation.

FIG. 16. Short posterior extremity in an adult trypanosome.

FIGS. 17 to 19. Trypanosomes with highly developed undulating membrane. Compare with figures 11 and 13.

FIG. 20. *Trypanosoma lewisi* with a rudimentary blepharoplast. Only the dot at the root of the flagellum represents the blepharoplast. The dark line is the result of heavy staining of the flagellum crossing the body of the organism. Compare with figure 11.

FIG. 21. An ablepharoplastic trypanosome.

FIG. 22. An ablepharoplastic trypanosome with an elongated posterior extremity.

ON CERTAIN SPONTANEOUS CHICKEN TUMORS AS MANIFESTATIONS OF A SINGLE DISEASE.

I. Spindle-Celled Sarcomata Rifted with Blood Sinuses.*

By PEYTON ROUS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Plates 69 to 71.

Recently three transplantable chicken tumors distinct in character have been found to have a filterable cause.[1] The differences between these tumors are traceable to differences in the causative agents. Each agent gives rise in normal fowls to tumors of the sort from which it was isolated by filtration, and to tumors of this sort only. For example, the agent derived from a transplantable osteochondrosarcoma gives rise to sarcomatous tumors in which cartilage and bone are laid down. Certain minor variations, it is true, do occur in each tumor strain as intercurrent phenomena. The cells of the sarcoma known in our laboratory as Chicken Tumor I are, in some chickens, of very attenuated spindle form, again oat-shaped or almost round, again interspersed with sarcomatous giant cells; and the course of the disease varies somewhat in individual fowls. But the growth is always a spindle-celled sarcoma, and its modifications are not greater than those observed in certain rat and mouse tumors propagated only by transplantation and dependent on the survival of a single race of cells. Attempts to bring about variations by injuring the filterable agent have been unsuccessful, as have attempts to make it affect epithelium.

There is good ground to suppose that other tumors of the fowl besides those already studied are caused by filterable agents. The range in structure and behavior among chicken tumors is very wide.

* Received for publication, April 20, 1914.

[1] Rous, P., *Jour. Am. Med. Assn.*, 1911, lvi, 198. Rous, P., and Murphy, Jas. B., *Jour. Exper. Med.*, 1914, xix, 52.

Even when composed of cells of similar origin they often exhibit, like mammalian growths, a strikingly various structure and course. Must one suppose a distinct causative agent not only for each type of neoplasm as determined morphologically, but for the almost infinite number of variations in structure and behavior of such types? The present article and the one following it deal with this point. Briefly, it has been found that two spontaneous chicken tumors recently transplanted have each given rise to neoplasms identical in composite behavior with a tumor strain already under propagation. As will be shown in the present paper, the spontaneous tumor known as Chicken Tumor 38 of our series, seems to be a manifestation of a disease-complex already reported upon and known as Chicken Tumor 18.[2] This latter growth is a spindle-celled sarcoma, rifted in a characteristic manner with blood sinuses and tending to metastasize to the muscles, especially in the neighborhood of joints.

THE SPONTANEOUS TUMORS.

The spontaneous tumor No. 38 resembled the spontaneous tumor No. 18 only in the fact that it was a growth composed of spindle cells of connective tissue origin. The fowl carrying it was a well grown but emaciated Plymouth Rock hen. It was brought to the laboratory while yet alive. The irregular tumor mass, situated in the subcutaneous tissue between the left leg and the body, measured 10 by 6 by 5 centimeters, was imperfectly encapsulated, attached to the sheath of the thigh muscles, and just beginning to involve the skin. Strands extended between the leg muscles into the drumstick. At the center of the mass was a cavity with ragged walls, containing about forty cubic centimeters of clear, straw-colored fluid. The tumor tissue was finely striated, pinkish white, rather soft, and varied with many irregular, translucent areas of colliquation. There were no metastases. Histologically the growth was composed of strands of attenuated spindle cells with much collagen, sometimes in the form of ribbons (figure 1). A few round cells were scattered here and there in the growth. There was very little resemblance to the spontaneous tumor No. 18. This latter occurred

[2] Rous, P., and Lange, L. B., *Jour. Exper. Med.*, 1913, xviii, 651.

in the gizzard of a brown Leghorn fowl and metastasized to several points in the skeletal muscles. Both the primary and secondary tumors consisted of a very regular spindle-celled tissue, rifted to an extraordinary degree with blood sinuses into which the growth showed a tendency to extend, with result in an intracanalicular arrangement.

The fowl carrying Chicken Tumor 38 was killed and bits of the neoplastic tissue were implanted in the breast muscle of two normal Plymouth Rock fowls, in both of which a growth slowly developed. With repeated passage the tumor's rate of growth has increased somewhat, but like No. 18 it is still much less malignant than the simple spindle-celled sarcomata, Nos. 1 and 43. It is now growing in its fourth successive series of hosts. A filterable agent causing it, distinct from the tissue cells, has been demonstrated by three methods; namely, by drying, by glycerination, and by filtration through Berkefeld cylinders impermeable to small bacteria. The findings compared with those in the case of No. 18 are, briefly, as follows:—

COMPARISON OF THE TUMOR STRAINS.

Both growths, whether obtained by transplantation or by the action of the filterable agent as such, are, in the gross, solid, pinkish white, unencapsulated, firm, and markedly resistant to the knife. When growing in voluntary muscle they tend to bind the fibers and limit motion, a feature not observed in the case of the simple spindle-celled sarcomata already mentioned. Both growths are composed of attenuated spindle cells arranged, often very regularly, in bundles or strands with much collagen which is usually in the form of bands or ribbons (figure 2). Giant cells are not present. The rifting with blood sinuses, which was so important a feature of the earlier generations of Chicken Tumor 18, is now only occasionally seen in this growth. Absent at first from Chicken Tumor 38, it has recently been met with in several cases (figure 3). Histologically the two growths are at present practically indistinguishable.

Chicken Tumor 18 in its earlier generations showed a notable tendency to metastasize to the skeletal muscles, especially in the neighborhood of joints. The lungs, heart, and liver were affected

sometimes, though rarely. Among nine fowls which have thus far died of Tumor 38, one had secondary tumors in lung, liver, and gizzard. In three cases there were metastases in the skeletal muscles. The simple, spindle-celled sarcomata, Nos. 1 and 43, have never shown this feature. Of the three instances referred to, one had a nodule in the wing, a second both wing and leg metastases, while in the third the involvement was widespread. A small nodule was present in the lung, larger secondary growths in the muscles of the neck, legs, and hip, and a series of coalescing masses connected the junctions of the sternal and vertebral ribs, forming what may be called a neoplastic rosary (figure 5). The whole condition closely resembled that in a fowl which died of tumors produced by the injection of a filtrate of Chicken Tumor 18.[3]

Tumor 18, though spontaneous in a brown Leghorn fowl, grows much better in the Plymouth Rock variety, a fact only recently determined and still to be reported upon in full. Chicken Tumor 38 likewise succeeds better in Plymouth Rocks than in brown Leghorns. But the two cases are hardly to be compared, for Tumor 38 occurred spontaneously in a Plymouth Rock fowl, and, from what is known of the laws governing transplantation, might be expected to succeed best in hosts of this sort, as indeed it does. Tumor 18 acts against the rule, growing better in hosts of an alien variety.

THE FILTERABLE AGENTS.

A causative agent for Chicken Tumor 18, as distinct from the cells, has been demonstrated only by filtration experiments. The dried or glycerinated tumor tissue is incapable of causing the growth. Tissue of No. 38, dried or glycerinated, gives rise to the tumor in a considerable percentage of normal fowls injected, and this within a few weeks. The Berkefeld filtrate of an extract of the growth acts almost as quickly. Filtrates derived from No. 18, on the other hand, seldom cause a tumor until several months after the injection. The differences in resistance and activity of the causative agents, as thus indicated, are the only points of dissimilarity between the tumors at present.

[3] Rous and Lange, *loc. cit.*

CHICKEN TUMOR 27.

All in all, the findings give one good reason to suppose that the spontaneous chicken tumors, Nos. 18 and 38, are different manifestations of a single disease-complex. It has seemed possible that other expressions of this complex might be present among our forty-five spontaneous neoplasms of the fowl. A search shows that the growth known as No. 27 is probably such a case. The host, a brown Leghorn hen, had several large lumps in the muscles of the wings and legs which limited motion markedly, a small nodule in the gizzard, and a number of raised, sharply defined, plateau-like masses in the skin, some deeply pitted with feather follicles and one of them ulcerated. It was impossible to say which growth was primary. All consisted of a close textured, finely striated, firm, pink, sarcomatous tissue. At the time, the case appeared unique, and indeed among the spontaneous growths subsequently obtained none has given a similar picture. But among the many fowls dying of transplantation tumors of No. 18,—now in its eleventh successive series of hosts,—a single instance closely resembling that of No. 27 has been met with. The fowl, of the second transplantation generation, is mentioned in a previous article.[4] The discoid masses in the skin consisted, as in the case of Tumor 27, of a sharply defined, nearly homogeneous, spindle-celled, sarcomatous tissue in the looser layers of the corium, the masses in the muscle of the same sarcoma arranged for the most part in the familiar intracanalicular pattern (figure 4). Unfortunately no adequate attempt was made to transplant Tumor 27. The other spontaneous chicken tumors do not suggest, even remotely, the disease-complex of Nos. 18 and 38.

SUMMARY.

Two spontaneous chicken tumors, unlike in several important respects, have given rise on transplantation to neoplasms of identical character. The spontaneous growth, No. 18, situated in the gizzard, was a spindle-celled sarcoma rifted with blood sinuses into which it extended, with result in what may be described as an intra-

[4] Rous and Lange, *loc. cit.*

canalicular pattern. The metastases, which were in the voluntary muscles, showed the same peculiar structure. Tumor 38, occurring in the subcutaneous tissue of the groin, was a solid, spindle-celled sarcoma of rather close texture, with few blood vessels. Here and there were small areas of softening, and at its center was a large degeneration cyst with ragged walls, containing a clear fluid. There were no metastases. The transplantation tumors from both growths have been characterized by slow growth, tendency to metastasize to the skeletal muscles without involvement of the lungs, and a structure which at one time is that of a very regular spindle-celled sarcoma containing many bands and ribbons of collagen, and at another that of a sarcoma rifted with blood sinuses like the spontaneous tumor No. 18. At present the two strains are practically indistinguishable in appearance and general behavior. Both are caused by filterable agents. The agent causing No. 38, unlike that causing No. 18, retains its activity in tumor tissue which has been dried or glycerinated; and in a Berkefeld filtrate it is much the more active in causing tumors. These differences can hardly be thought of as constituting a fundamental distinction between agents which, to judge from their effects, are almost undoubtedly different strains of a single disease cause.

That chicken tumors of markedly different type have different filterable agents as their cause has been proved by experiments already reported. The present findings make it probable that, within certain limits, tumors of rather various character may be dependent upon a single agent. This assumption greatly simplifies the etiological problem. But the truth of the assumption for other instances than those described in the present article can only be determined by the study and comparison in many hosts of the disease-complexes of which each spontaneous chicken tumor is to be considered as an individual expression.

EXPLANATION OF PLATES.[5]

PLATE 69.

FIG. 1. Section of the spontaneous tumor No. 38. It is composed of spindle cells in strands, with abundant collagen. There is some round-celled infiltration.

FIG. 2. A solid growth of the third generation of transplants. The heavy black spots are artefacts.

PLATE 70.

FIG. 3. Portion of a growth that resulted from the injection into a normal fowl of tumor tissue that had been dried while frozen. The rifting with blood sinuses here shown has been found in several transplantation growths as well.

FIG. 4. Spontaneous Tumor 27. Section of one of the growths in the skeletal muscles.

PLATE 71.

FIG. 5. Secondary growths in a fowl of the first transplantation series of Tumor 38. The primary growths were situated in the pectoral muscles. They have been removed with the sternum except for a small portion of that on the left (*A*). There are metastases in both legs near the knee (*B*), in the neck muscles (*C*), and in the muscles within the bony trunk (*D*). A number of nodules coalescing into a thick cord (*E*) connect the junctions of the sternal and vertebral ribs.

[5] The microscopic sections were stained with methylene blue and eosin. The illustrations should be compared with those of Chicken Tumor 18 (Rous and Lange, *loc. cit.*).

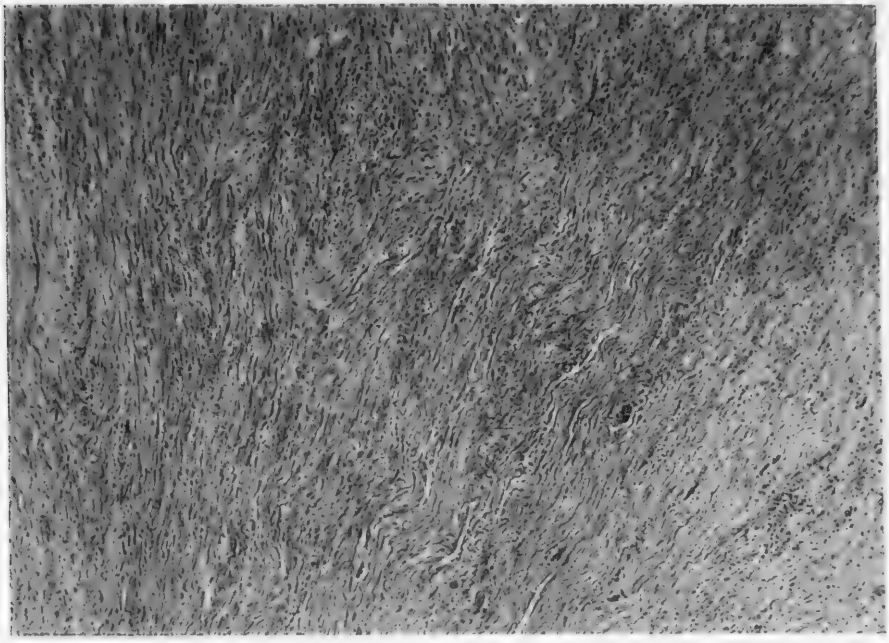

FIG. 1.

FIG. 2.

(Rous: Spontaneous Chicken Tumors.)

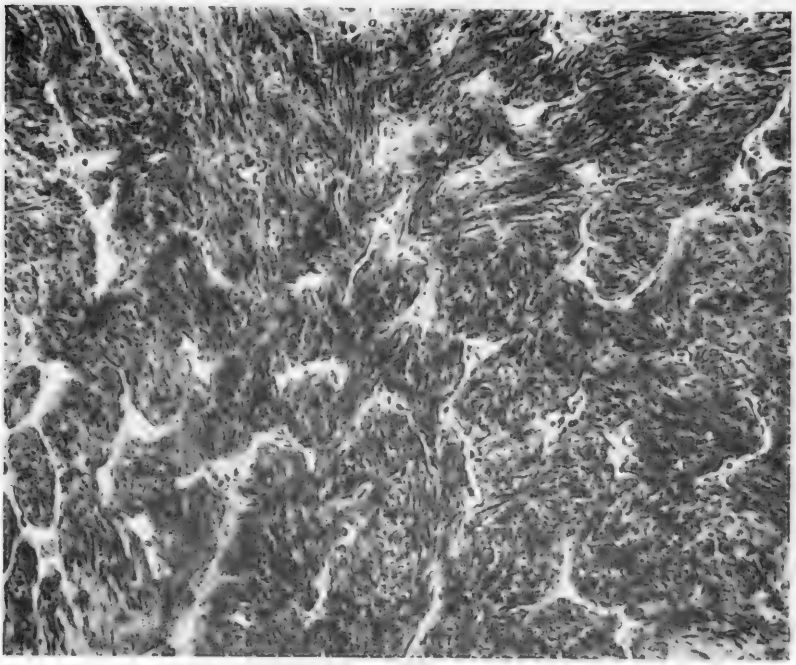

FIG. 3

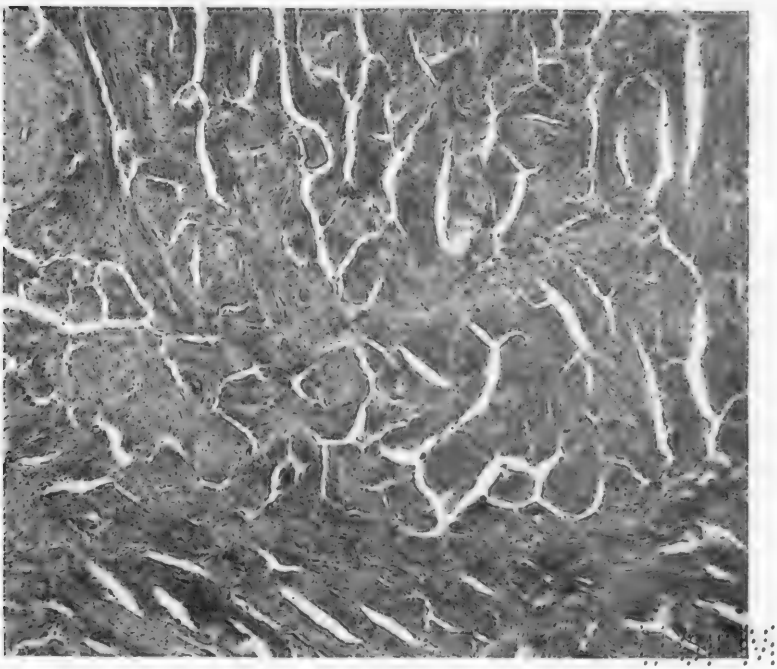

FIG. 4.

(ROUS: Spontaneous Chicken Tumors.)

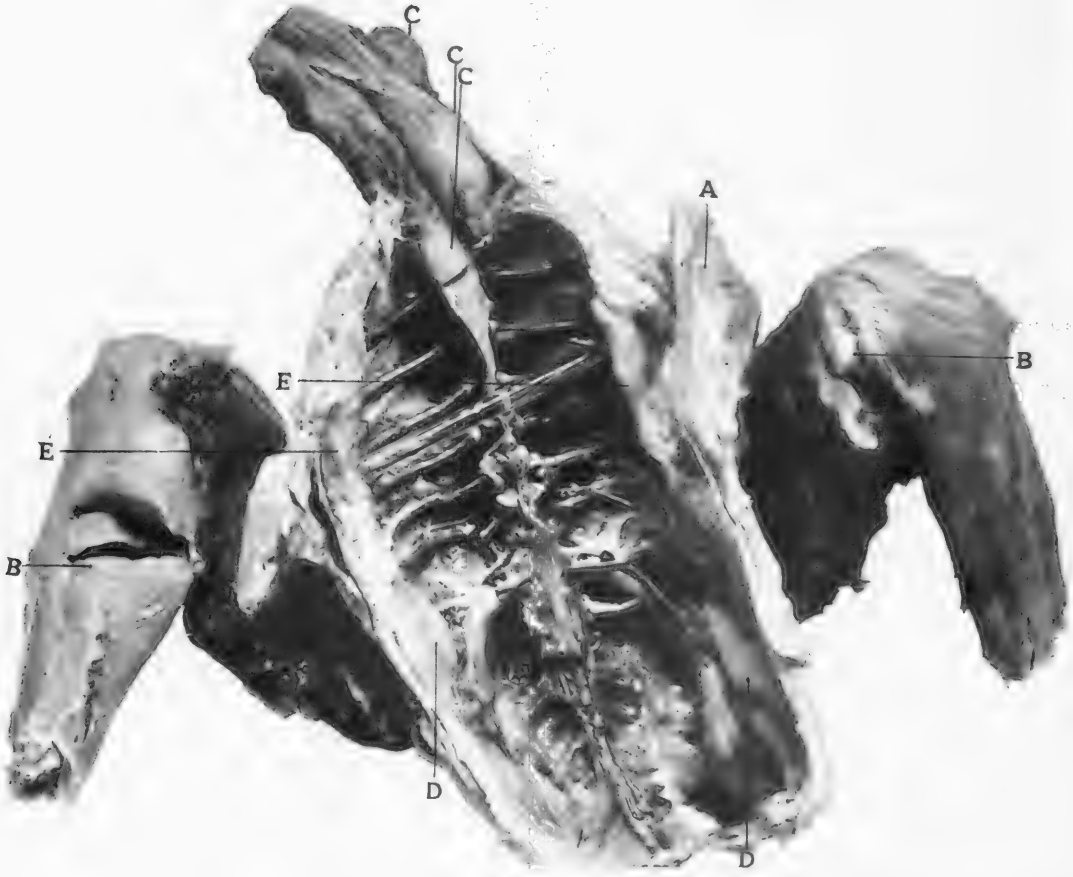

FIG. 3.

(Rous: Spontaneous Chicken Tumors.)

U of M

ON CERTAIN SPONTANEOUS CHICKEN TUMORS AS MANIFESTATIONS OF A SINGLE DISEASE.

II. Simple Spindle-Celled Sarcomata.*

By LINDA B. LANGE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Plates 72 to 74.

Among the spontaneous chicken tumors recently brought to this laboratory there have been two spindle-celled sarcomata that have yielded, on transplantation, neoplasms similar respectively to two strains already under propagation. The resemblance of the growths derived from Chicken Tumor 38 to those derived from Chicken Tumor 18 is taken up in the preceding article.[1] They are spindle-celled sarcomata of protean character, often rifted with blood sinuses in a characteristic manner and showing a tendency to metastasize to the voluntary muscles. The subject of the present paper is Chicken Tumor 43, a simple spindle-celled sarcoma, apparently identical with Chicken Tumor 1.

The spontaneous tumor No. 38 differed considerably from the spontaneous tumor No. 18, and only after the growths had been observed in many hosts was their close similarity realized. Tumor 43, on the other hand, in its original form strikingly suggested Tumor 1, and the transplantation growths are practically identical with those of the latter. Both are produced by a filterable agent.

GROSS CHARACTERISTICS.

The original Chicken Tumor 43 occurred in a Plymouth Rock hen as a large nodular mass in the substance of the pectoral muscle, loosely attached to the lower end of the sternal keel, but not in-

* Received for publication, April 20, 1914.

[1] Rous, P., *Jour. Exper. Med.*, 1914, xix, 570.

volving the skin. The mass was fairly well defined, but devoid of a capsule. On incision the tumor tissue was, for the greater part, smooth, greyish pink, and firm, traversed by a few large blood sinuses, and well nourished throughout.

Pieces of the fresh tissue were inoculated into the pectoral muscles of two Plymouth Rock fowls by means of small trocars, the method employed also in subsequent transfers. The tumor grew in one of the two fowls. The rate of growth was slow at first, but on transplantation the malignancy increased rapidly, and in the fourth tumor generation the number of takes reached 100 per cent. The rate of growth as measured by the longest diameter was three times as rapid in the eighth as in the first generation. As contrasted with the firm almost gristly tissue of the earlier growths the more malignant tumors of later generations have been translucent and friable, and wet with a mucinous fluid. Hemorrhage into the tumors is frequent. In resistant fowls, on the other hand, the growth is firm, dense, nodular, and may undergo liquefaction resulting in cysts containing a clear mucinous fluid. With the progressive enlargement of the tumors the host emaciates, becomes cyanotic, and finally dies in coma.

Metastases were found in the original fowl in the heart and lungs, but not again until the fourth transplantation generation when they were also situated in the heart and lungs. Metastases have since been fairly frequent. They usually occur in the heart, lungs, and liver, less often in the spleen and kidney (figures 1 and 2). Implantation tumors on the heart and liver from a tumor growing through the body wall have been observed once.

MICROSCOPIC FINDINGS.

The tissue of the original growth is composed for the most part of slender spindle cells of somewhat irregular size, with pale, oval, vesicular nuclei often containing elongated or double nucleoli. Mitotic figures are fairly frequent. Scattered through the tissue are a few giant cells (figure 3). In some areas the cells are plumper and irregularly oval. The tissue structure varies, being very compact in some places and loosely meshed in others.

This picture has been fairly constant. Among the transplantation growths giant cells have been infrequent and the tumors have presented the general characters of a simple, spindle-celled sarcoma. In hosts relatively resistant, as shown by the behavior of the tumor, accumulations of small round cells are found, especially at the edge of the neoplastic tissue. The vigorously growing tumors are composed of spindle cells fairly uniform in size, shape, and arrangement. At the edge of the tumors there is practically no cellular reaction. When very malignant the tumor invades the muscle, not only by growing between the muscle bundles and fibers, but by penetrating the sarcolemma and replacing the muscle substance directly (figure 4). The metastases are histologically identical with the primary tumors.

ETIOLOGY.

Bacteriological cultures from the tumor tissue on the ordinary media have remained sterile under aerobic and anaerobic conditions. A causative agent separable from the tumor cells has, however, been demonstrated. The clear fluid obtained by filtering a thin suspension in Ringer solution of the finely ground tumor through Berkefeld filters holding back a test bacterium at the same filtration is capable of giving rise to tumors in normal fowls. These filtration tumors are identical with those from which the tissue for the emulsion was obtained. Tissue ground, frozen, and dried *in vacuo* over sulphuric acid, made up to the original bulk with distilled water, and injected into normal chickens, likewise causes tumors. Finally, the opalescent fluid obtained by centrifugalizing a thin suspension of the ground tissue in Ringer's solution may be mixed with glycerin and kept at 5° C. for many days without losing its ability to cause tumors. Mixtures containing 80 per cent. glycerin tested after ten days, and those containing 50 per cent. glycerin tested after twenty-one days were still active. Tumors arose from two to three weeks after inoculation of the material. As with the other chicken tumors, no case of cage infection has ever occurred.

RESEMBLANCE TO CHICKEN TUMOR I.

The spontaneous Chicken Tumor 43 and its transplanted growths closely resemble those of the strain known as Chicken Tumor 1.[2] They are of similar gross and microscopic structure, run the same course, metastasize, in general, to the same organs and have the same general action on the host. In appearance and behavior they are, indeed, indistinguishable. An etiological agent distinct from the living cells is easily demonstrated for both tumors by filtration, desiccation, and glycerination. In the absence of definite experiments upon the point it cannot be affirmed that both tumors have the same cause, yet a closer parallelism between two strains of the same disease obtained by transfer from separate instances occurring in nature could hardly be looked for. In this connection it is of interest to note that Chicken Tumor 13 (figure 5), a growth arising in the connective tissue of the foot, has some resemblance in its histology to Nos. 1 and 43, though it may not with certainty be classed with them. The attempt to propagate No. 13 was unsuccessful and nothing can be said as to its etiology.

SUMMARY.

The forty-third spontaneous chicken tumor received at this laboratory strikingly resembles the first and has given rise on transplantation to an entirely similar series of neoplasms. Tumors of both strains are due to a filterable agent which remains active in the dried or glycerinated tissue.

EXPLANATION OF PLATES.[3]

PLATE 72.

FIG. 1. Characteristic tumor filling out the left breast of the fowl. The right breast shows the emaciated state of the fowl. Metastases can be seen in the heart, lungs, and liver, as indicated by the arrows. This tumor arose from glycerinated tissue.

FIG. 2. Metastasis in the heart from a tumor of the eighth transplantation generation. There is a complete absence of a cellular reaction about the growth.

PLATE 73.

FIG. 3. Section of the original growth showing subcutaneous spindle-celled neoplasm with scattered giant cells.

FIG. 4. Tumor of the sixth transplantation generation invading striated muscle. The muscle fibers are directly replaced by tumor cells.

PLATE 74.

FIG. 5. Section of Chicken Tumor 13.

[2] Rous, P., *Jour. Exper. Med.*, 1910, xii, 696; 1911, xiii, 397.

[3] The microscopic sections were stained with methylene blue and eosin.

FIG. 1.

(Lange: Spontaneous Chicken Tumors.)

FIG. 2.

FIG. 3.

(Lange: Spontaneous Chicken Tumors.)

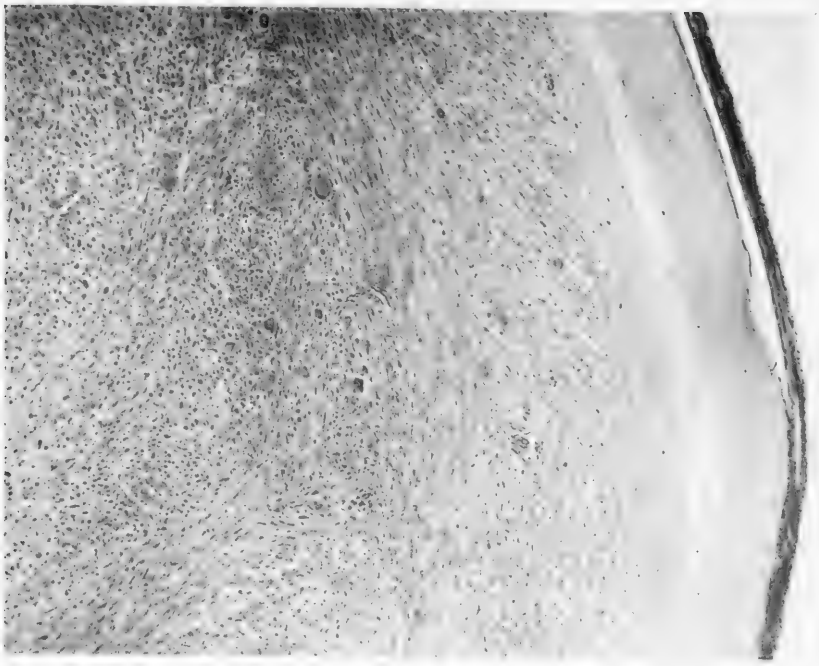

FIG. 4.

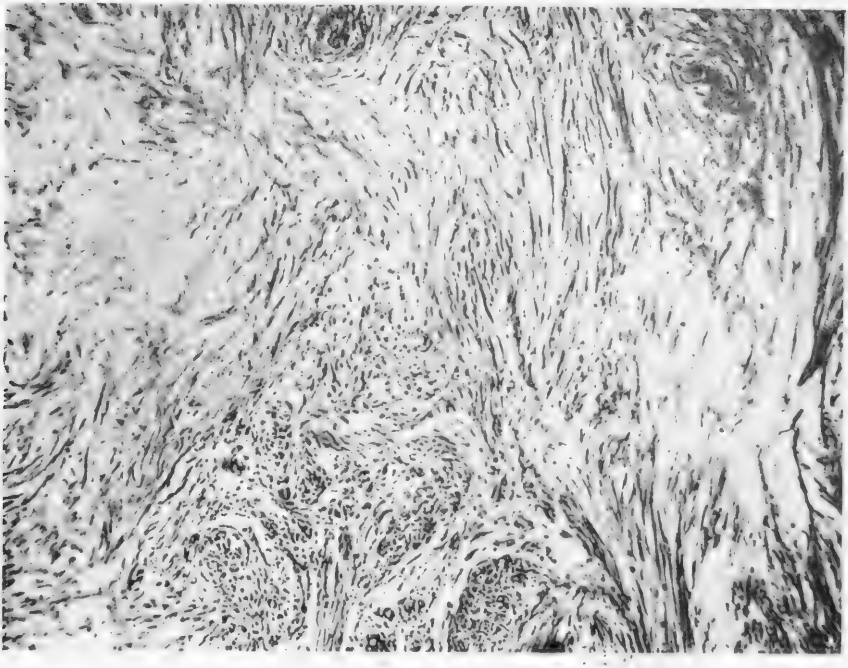

FIG. 5.

(Lange: Spontaneous Chicken Tumors.)

THE RECOGNITION OF THE CHOLERA VIBRIO.*[1]

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(From the State of New York Health Officer's Department, Rosebank.)

Curved organisms resembling in many ways the cholera vibrio of Koch are known to occur widely in nature, chiefly as saprophytes externally, and also incidentally in one or more of the fluid secretions of the body. It is only during the prevalence of cholera epidemics that the presence of a comma-shaped organism in the intestinal contents of a person sick with an acute diarrhea may be regarded upon morphology alone as strong presumptive evidence of an infection by the specific vibrio.

THE CHOLERA-LIKE VIBRIOS.

Saprophytic cholera-like vibrios have been found in healthy persons from infected localities, and in those suffering from diseases other than cholera, and in the waters of rivers and wells of cholera-infected districts. The morphology and cultural appearances of these vibrios at times closely approximate those of the cholera organism, differences being usually of degree only; so that we may say with the exception of the negative reaction with anticholera sera, the cholera and non-cholera vibrios appear closely related to a remarkable extent. For this reason the gross appearances, which originally were considered by Koch so distinctive as to make possible a definite diagnosis of cholera alone, have been found to be the characteristic features of a large class of bacteria, many members of which have been found to be able to sustain existence in the fluids of the body as well as to lead a saprophytic life outside.

As far as we know, the cholera-like vibrios are non-pathogenic to man, but how far it is justifiable to assume that all non-aggluti-

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[1] In the preparation of this article I have to acknowledge the kind assistance of Dr. Meriggio Serrati, of the Italian Royal Navy.

nating vibrios are invariably of a permanent harmless saprophytic type has been for some time a question of conjecture, especially in those instances where cholera cases have occurred in the vicinity within a recent period of time, and where infection by an attenuated variety of the cholera vibrio cannot be ruled out altogether as a possibility. It is certain that wherever cholera cases have occurred proper search has revealed the concurrent existence of the non-agglutinating vibrios.

CHOLERA-LIKE VIBRIOS IN CHOLERA EPIDEMICS.

Gottschlich, at Tor in Egypt in 1905–1906, isolated 48 non-agglutinating vibrios from cases of acute dysentery; and in 1911 on examining 1,160 pilgrims he found 31 cholera bacillus carriers and 23 carriers of non-agglutinating vibrios.

MacLaughlin,[2] at Manila in 1908, made a bacteriological examination of 376 cholera contacts, and from them isolated 27 cholera vibrios, and 46 non-agglutinating cholera-like vibrios.

The presence of non-agglutinating vibrios was particularly remarked during the months preceding the cholera epidemic in St. Petersburg in 1910, during which time cases of gastro-enteritis occurred with great frequency, and from the dejecta of some of the patients vibrios were isolated which did not agglutinate with specific anticholera sera and which had therefore to be classed as of a non-cholera nature. The etiological relation between the cholera-like organisms and the concurrent disease, although significant, was not regarded as definitely established.

VARIATIONS IN CHOLERA STRAINS.

It has been frequently noted that the Asiatic vibrio possesses to a marked degree the tendency of the group to undergo polymorphism, the development of atypical forms, which may occur in recently isolated strains from cholera cases and carriers, as well as in old laboratory cultures. Notable differences in morphology have been observed in cholera vibrios isolated from different cholera cases in the same epidemic.

During the epidemic in St. Petersburg in 1909–1910, Horowitz[3] noticed that the morphology of the cholera vibrios was not constant, and that under varying conditions the same vibrio could present such divergences from type as to become unrecognizable. The tendency to modification from type occurs also in the cultural properties which have been found to vary considerably in different

[2] MacLaughlin, *Philippine Hosp. Rep.*, 1912, xxvii, 381.

[3] Horowitz, A., *Bull. de l'Inst. Pasteur*, 1911, ix, 786.

strains, although some of these may persist more than others. The ability to liquefy gelatin, for instance, is generally present in the cholera vibrio, as it is in most of the cholera-like vibrios.[4] Virulence upon animals is not a constant property and may be altogether absent in recently isolated typical cultures from cholera cases.

Zlatogoroff[5] states that the virulence to guinea pigs of some non-agglutinating vibrios exceeds that of the cholera vibrio. The indol reaction has proved to be so uncertain that it is of little value in a final differentiation. Liefmann and Nieter[6] and others have shown that cholera vibrios possess hemolytic action upon blood media, as well as do the other members of this class of bacteria.

SERUM DIAGNOSIS.

It is by reason of these resemblances in form and culture between the cholera and cholera-like vibrios, that certain biological properties have become of importance for the purpose of diagnosis, and by means of these it is generally assumed that we have been able to recognize fundamental differences in the behavior of the typical Asiatic organism and other members of the same group.

It is well known that the presence of the specific vibrio in the body of cholera-patients and of highly immunized animals causes strong activation of those tissues which bring about the formation of cellular antibodies, leading to the phenomena of agglutination, bacteriolysis, complement deviation, and serum precipitation, when the specific antigen is added to the separated immune serum. Of all pathogenic organisms the cholera vibrio is the most susceptible to the action of serum antibodies, positive agglutination taking place in extreme dilutions of the antiserum (1 to 10,000 and 1 to 40,000); and similarly with a strong bactericidal serum, bacteriolysis may be observed by Bordet's method *in vitro* in high dilutions, or *in vivo* by means of Pfeiffer's method. The reactions with the anticholera serum may be said to be specific for the cholera vibrio, and it has been considered that when we are dealing with a typical cholera culture these tests enable us to come to a definite conclusion as to whether or not we have the specific organism or a cholera-like vibrio before us. By the serum reactions, epidemic vibrios have been divided into two classes: the first includes those which agglutinate

[4] Craster, C. V., *Jour. Infect. Dis.*, 1913, xii, 472.
[5] Zlatogoroff, S. J., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1908, xlviii, 684.
[6] Liefmann, H., and Nieter, A., *Med. Klin.*, 1906, ii, 254.

with a specific serum in high dilution, give a positive Pfeiffer reaction, and are capable of producing in immunized animals a serum which will agglutinate in a specific manner other cholera cultures (cross agglutinations); the second class includes all the cholera-like vibrios which give a negative agglutination with anticholera sera, or agglutinate in low dilution only 1 to 10 and 1 to 50, and in which the Pfeiffer reaction and cross agglutination are generally negative. The existence of a third class presumes a condition of the cholera vibrio in which certain biological properties have become latent.

THE DEVELOPMENT OF AGGLUTINATION IN CHOLERA-LIKE VIBRIOS.

The virulence of cholera cultures is known to lapse with long continued cultivation, and agglutination power to become considerably modified under the same condition, unless maintained by repeated animal inoculation.

The possibility of a temporary disappearance of the agglutinating power of the cholera vibrio under unusual conditions of environment and association with other bacteria, outside the animal body, and its subsequent reëstablishment by cultural and inoculation methods was first suggested by Zlatogoroff.[7] In the course of a study of eighteen different strains of non-agglutinating vibrios, isolated from the water of rivers and wells during a cholera epidemic in Saratow in 1908, he noted, after a month's culture upon ordinary laboratory media, a spontaneous development of agglutination with anticholera sera on the part of several of the vibrio cultures, which had previously given a negative reaction. Suspecting from this occurrence that with the remainder he might possibly have to deal with cholera vibrios that had become more or less changed by environmental conditions, he originated the following procedures for influencing the return of agglutinating properties in cultures of non-agglutinating vibrios: (1) repeated daily cultivation upon fresh alkaline beef agar with alternate incubation at 37° C. and cool storage at 16° to 18° C; (2) weekly passage through the peritoneal cavity of a series of guinea pigs.

Because of the slight virulence of the majority of the water vibrios, it became necessary to increase this property before successful passage through animals could be carried out, and this was accomplished by injecting with the vibrio cultures a certain quantity of dead typhoid bacilli, colon bacilli, or streptococci. The result of this treatment was that at the end of fifty-four generations, out of the eighteen vibrio strains which did not previously agglutinate ten became possessed of full agglutinating properties with an anticholera serum, in dilution of 1:10,000 and 1:20,000, a very high agglutinating titer. To establish further the cholera nature of his vibrios Zlatogoroff controlled his results by means of the complement fixation test of Bordet, in each case obtaining a con-

[7] Zlatogoroff, S. J., *loc. cit.*

firmation of the positive results with the agglutination reaction. As all these vibrio strains which eventually developed specific agglutination were isolated from the water of rivers and wells in Saratow, Zlatogoroff looked upon them as true cholera vibrios which had become weakened by a direct passage out of the agglutinogen from the bodies of the vibrios into the water, due either to the phenomenon of osmosis or to the action of the chemical constituents of the water upon the external capsule of the organism, changing in this way their biological reaction from a parasitic to a saprophytic type. To test further the theory of the loss of agglutination power of vibrios in water Zlatogoroff allowed a suspension of the cholera vibrio to remain in distilled water for seven days at room temperature. After centrifugalization and washing several times in distilled water, the agglutinating titer was found to have fallen from 1 : 5,000 to 1 : 1,000, and further treatment on these lines reduced the agglutination titer as low as 1 : 300. Barrenscheen,[8] repeating the experiments of Zlatogoroff, cultivated the cholera vibrio, which normally agglutinated in a dilution of 1 : 40,000, for eight days in distilled water, and found the agglutination titer reduced to 1 : 2,000, and after seven days more to 1 : 200. Both Zlatogoroff and Barrenscheen noted that when the cholera vibrio was kept for some days in distilled water there occurred a passing out of a substance (agglutinogen) from the bacterial bodies, which was shown by the appearance of an opalescence and later a flocculent precipitate in the centrifugalized culture liquid, after the addition of cholera immune serum. Horowitz[9] states that about 4 per cent. of vibrios isolated from actual cholera sources eventually regained agglutinating power, either spontaneously or with the use of Metchnikoff's method by cultural symbiosis with the yellow sarcinæ. In confirming the cholera nature of the vibrios by cross agglutinations, Horowitz found that the antisera from animals immunized against the same vibrios did not give the same reactions with different strains of cholera vibrios. At the same time some vibrios which gave a negative agglutination with an anticholera serum were able to produce in animals a serum which had a strong agglutinating reaction upon typical cholera cultures. In the case of other vibrios isolated from the stools of cholera convalescents the cross agglutination was negative at first, but later the appearance of a positive reaction with the specific serum and the Pfeiffer phenomenon proved their cholera nature. Horowitz concludes that among the cholera vibrios were certain strains presenting biological differences which evidently indicate some form of evolutionary change brought about by the reaction of the living cell to its altered environment. Carapelle,[10] in examining a series of sixteen vibrio cultures isolated from water in and around the city of Palermo during the cholera epidemic of 1911, in seven of which direct fecal infection of the water was evident, noted that the specific agglutination was negative or only positive in extremely low dilution of the antiserum, although the bacteriolytic reaction *in vitro* and complement deviation tests pointed to their cholera nature. Twelve cases of cholera occurring at this time in a hospital which had been carefully guarded against outside infection, and from the household water of which non-agglutinating

[8] Barrenscheen, H., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1909, l, 261.
[9] Horowitz, A., *loc. cit.*
[10] Carapelle, *Ann. d'ig. sper.*, 1912, xxii, 497.

vibrios had been isolated, caused this observer to suspect that these vibrios might be of a cholera nature. He accordingly subjected the non-agglutinating vibrios to the procedures suggested by Zlatogoroff, and by passage through a number of guinea pigs alone a high agglutinating titer was developed in all the vibrio cultures (1:4,000). The virulence was also greatly enhanced and bacteriolysis *in vitro* became much more complete. By immunizing animals with the vibrio cultures he was able to obtain antisera which agglutinated typical cholera vibrios in maximum dilution. Carapelle thus corroborates the conclusions of Zlatogoroff that non-agglutinating vibrios obtained from water and subsequently developing agglutination with anticholera sera were really true cholera vibrios which had temporarily lost this property. In order to prove his conclusions of the loss of agglutination in water he exposed suspensions of cholera vibrios in sterile water contained in Berkefeld candles to the action of running water so that the water could wash the vibrios in suspension. After twenty-two days the agglutinating power of the cholera vibrios contained in the candles had been reduced to 1:100.

In a former communication[11] I described the properties of a number of cholera-like vibrios which were isolated from rectal swab cultures during a cholera outbreak at Quarantine, N. Y., in 1911.[12] All were obtained from persons free from any disease, the majority having been isolated some weeks after cases of cholera had ceased to arrive in the port. About 15 per cent. gave a modified positive indol reaction, and the hemolytic power upon blood agar was found to be variable for each strain. At that time the morphological and cultural differences discernible between the cholera and cholera-like vibrios were in many instances so slight that little importance could be attached to them for the purpose of differential diagnosis.

In the preliminary examination for specific agglutination, although negative results were obtained in moderately high dilutions of the antiserum, there were revealed at the time considerable differences in the reactions with many of the vibrio cultures, the differences varying from a complete negative result at any dilution to one showing moderate clumping at dilutions much below that of a typical cholera culture. This was looked upon at the time as due to the presence of group affinities. The Pfeiffer reactions carried out with the cholera-like cultures were found to be negative, and a series of cross agglutination tests with the cholera vibrio and antisera obtained by immunizing rabbits with cultures of the non-agglutinating vibrio gave unsatisfactory results on account of the low titer of the antisera produced.[13]

Some time later when the vibrio cultures had been kept upon laboratory media for several months an examination of the agglutination power showed that three of the cultures which had formerly agglutinated only in very low dilution of the antiserum now gave a positive reaction in slightly greater dilution (1 to 100). This re-

[11] Craster, *Jour. Infect. Dis., loc. cit.*

[12] Craster, *Jour. Am. Med. Assn.*, 1913, lxi, 2210.

[13] Craster, *Jour. Infect. Dis., loc. cit.*

sult, although not high enough to be looked upon as a specific agglutination, suggested the advantage of attempting to increase this property in the cultures and in others in which agglutinations had not so developed, upon the lines pointed out by Zlatogoroff and Carapelle. For this purpose fourteen vibrio cultures, including the three agglutinating strains, were selected. Table I shows that the

TABLE I.

Properties of Cholera-Like Vibrios.

No. of vibrio culture.	Gelatin liquefaction.	Fermentation.				Hemolysis.	Indol reaction.	Pathogenicity for guinea pigs.
		Saccharose.	Glucose.	Lactose.	Maltose.			
98	+	+	+	−	+	−	+	−
103	+	+	+	−	+	+	+	−
109	+	+	+	+	+	+	−	−
125	+	+	+	−	+	−	−	−
151	+	+	+	−	−	+	−	−
219	+	+	+	+	+	+	+	−
269[14]	+	+	+	−	+	+	−	−
899	+	+	+	−	+	+	−	−
2464[14]	+	+	+	−	+	−	−	−
3064	+	+	+	+	+	−	−	−
3832	+	+	+	−	−	+	+	−
5947	+	+	+	+	+	+	−	−
5999	+	+	+	+	+	−	−	−
6061	+	+	+	−	+	+	+	−

Gelatin liquefaction after 72 hours. Hemolysis on rabbit blood agar. Pathogenicity tested with one whole agar slant culture upon a guinea pig weighing 250 gm.

cultural properties differ only slightly; hemolysis in rabbit blood agar is generally positive, the indol reaction is positive in a few cases, and pathogenicity to guinea pigs is absent in doses of one agar slant. The Pfeiffer reaction was negative for all.

At the commencement of experimental work vibrio cultures 103, 899, and 5,999 showed a positive agglutination with specific anticholera serum in dilution of 1 to 100 (table II). Cultures 151, 269, and 3,832 gave a similar reaction in dilutions of 1 to 40, and cultures 98 and 219, in dilutions of 1 to 20 only. The virulence being extremely low, special measures had to be taken to insure successful animal passage. This was attained in some cases by inject-

[14] Pigment former.

TABLE II.

Agglutination of Cholera-Like Vibrios with Anticholera Serum and Pfeiffer Reaction at Commencement of Experimental Work.

No. of vibrio culture.	Normal saline.	Normal horse serum.		Anticholera serum.					Pfeiffer test.
		1:10	1:40	1:20	1:40	1:100	1:500	1:1,000	
98	−	−	−	+	−	−	−	−	−
103	−	+	−	+	+	+	−	−	−
109	−	−	−	−	−	−	−	−	−
125	−	+	−	−	−	−	−	−	−
151	−	+	−	+	+	−	−	−	−
219	−	−	−	+	−	−	−	−	−
269	−	+	−	+	+	−	−	−	−
899	−	−	−	+	+	+	−	−	−
2464	−	−	−	−	−	−	−	−	−
3064	−	−	−	−	−	−	−	−	−
3832	−	+	−	+	+	−	−	−	−
5947	−	+	−	−	−	−	−	−	−
5999	−	−	−	+	+	+	−	−	−
6061	−	−	−	−	−	−	−	−	−

Nos. 103, 899, and 5,999 developed agglutination in 1:100 dilution of antiserum, in laboratory cultivation after isolation.

ing two or more agar slant cultures emulsified in normal saline at one time into the peritoneal cavity of a guinea pig weighing 250 grams. When this method failed cultures of dead typhoid bacilli or *Bacillus coli* were injected at the same time.

After serial passage through five guinea pigs, and daily cultivation upon fresh alkaline agar for seventy generations, considerable increase in the agglutinating power was observed to have taken place (table III). Of the three vibrio cultures formerly agglutinating in serum dilution of 1 to 100, Nos. 899 and 5,999 showed positive results in serum dilution 1 to 2,000, and No. 103 in a dilution of 1 to 1,000.

Vibrios 269 and 3,832 increased in agglutination from dilutions of 1 to 40 to 1 to 100; and cultures 219 and 98 from 1 to 20 to 1 to 400 and 1 to 100, respectively. The response of the agglutinating power to intensive cultivation and animal passage was progressive, although the limit of agglutination was reached in some instances by the second or third animal passage, further inoculation producing no increase in this property.

TABLE III.

Agglutination of Cholera-Like Vibrios with Anticholera Serum and Pfeiffer and Bordet Reactions after Intensive Cultivation and Passage through Guinea Pigs.

No. of vibrio culture.	Normal horse serum.		Anticholera serum.						Pfeiffer test.	Bordet test.
	1:10	1:40	1:20	1:40	1:100	1:400	1:1,000	1:2,000		
98	−	−	+	+	+	−	−	−	−	−
103	+	−	+	+	+	+	+	−	−	+
109	−	−	−	−	−	−	−	−	−	−
125	+	−	−	−	−	−	−	−	−	−
151	+	−	+	+	−	−	−	−	−	−
219	−	−	+	+	+	+	−	−	−	+
269	+	−	+	+	+	−	−	−	−	−
899	−	−	+	+	+	+	+	+	−	+
2464	−	−	−	−	−	−	−	−	−	−
3064	−	−	−	−	−	−	−	−	−	−
3832	+	−	+	+	+	−	−	−	−	−
5947	+	−	−	−	−	−	−	−	−	−
5999	−	−	+	+	+	+	+	+	−	+
6061	−	−	−	−	−	−	−	−	−	−

The virulence upon animals had not been increased to any great extent by these methods. The most virulent culture, No. 5,999, became pathogenic to a guinea pig weighing 250 grams in doses of one platinum loop of a twenty-four hour agar-grown culture, and the others required larger doses than this to produce death of a similar sized guinea pig in twenty-four hours. To this absence of high virulence must be ascribed the failure to give a positive Pfeiffer reaction which was negative for all the vibrio cultures at the end of the period of special treatment.

For this reason the effect of an anticholera bacteriolytic serum upon the agglutinating vibrio cultures was tested *in vitro* by the method of Bordet.[15] The specific bacteriolytic serum was diluted so that when added to a mixture of the bacterial emulsion and fresh guinea pig serum to provide alexin (complement), dilutions of the specific serum of 1 to 200, 1 to 500, and 1 to 1,000 were obtained. Controls were made with the same quantities of the bacterial emulsion and alexin, but without the specific serum.

Hanging drop preparations of these mixtures were made, and they were incubated at 37° C. and examined in two to three hours.

[15] Bordet, J., *Ann. de l'Inst. Pasteur*, 1895, ix, 462.

A positive result showed complete lysis of the vibrios with the immune serum; the controls showed actively motile organisms. If the results after this time were incomplete, a further examination was made in eighteen to twenty-four hours at 37° C.

By this method positive bacteriolysis was observed to occur with cultures 103, 219, 899, and 5,999 (table III) in serum dilution of 1 to 1,000. The remaining vibrio cultures gave negative results even in low dilutions of the serum (1 to 200). Control hanging drop tests with known cholera vibrio cultures were carried out at the same time.

If we consider the results obtained by these methods, it will be seen that certain vibrio cultures which formerly gave so slight an agglutination as to be considered negative, later reacted in a specific manner with an anticholera serum. Similarly a strong bactericidal serum exerts upon them a bacteriolytic power seen only in the case of true cholera vibrios. If it cannot be said definitely that these vibrio cultures are really cholera, we are at least justified in strongly suspecting their cholera nature until other means of proving their true character are found.

Upon this hypothesis it may be agreed that the cholera vibrio in the process of attenuation in the intestinal contents at times loses simultaneously with its ability to produce the disease the power of responding to circulating agglutinins. The loss of agglutination may be due to the production by the bacterial cell of a defensive ferment of an anti-agglutinating nature, probably a necessary function for continued existence in hostile body fluids. Upon removal to an environment away from these serum antibodies the necessity for the formation of defensive substances ceases to exist, and agglutination tendencies slowly return.

Experience has shown that the exact value to be placed upon tests depending for their accuracy upon the possession of biological properties rests upon the appreciation of the margin of error which must be allowed for in their proper manipulation. It is well understood that biological properties are not always constantly manifest and are subject to variation in the same bacterial strain,—such changes being dependent upon conditions of environment, such as cultural media and the presence of other bacteria. Changes of this

character are well exemplified in the modifications observed as taking place in the agglutinating property of *Bacillus typhosus,* an organism which is peculiarly susceptible to the action of agglutinating sera. Many observers have noted that some varieties of this bacillus after isolation from typhoid stools or from infected water have shown at first little or no tendency to agglutinate with an antityphoid serum, but that further cultivation upon laboratory media brings about strong agglutination properties. *Bacillus typhosus* loses its agglutinating property rapidly in water, but it may be restored by cultivation in suitable media. Gay and Claypole[16] have noted variations in the agglutinability of freshly isolated typhoid bacilli, especially the frequent failure of typhoid blood cultures to clump with the antiserum from an animal immunized by means of agar-grown typhoid cultures. A peculiar deviation of agglutination was induced by growing the agar-cultivated organism upon blood agar for two or three generations. These cultures would then agglutinate with the serum from an animal immunized against blood media cultures but not against that of an animal immunized against agar-grown cultures. It is clear then that in the case of *Bacillus typhosus* the property of agglutination with a specific serum may be absent under natural conditions in the stools of typhoid patients, or, if present, it may be modified by growth in different media.

The agglutination property of the cholera vibrio has also been found to become considerably modified or lost under experimental conditions when cultivated upon a medium composed of bouillon and anticholera serum,[17] and the same result may be brought about by the use of earth and water media.[18] Conversely non-agglutinating vibrios if subjected to cultural symbiosis with sarcinæ may develop at times specific agglutination with an anticholera serum.[19]

Great variation in the agglutination of cholera cultures was observed by Pottevin[20] in a study of 127 vibrio cultures isolated at Tor in Egypt in 1912–1913. The agglutination power of many of the

[16] Gay, F. P., and Claypole, E. J., *Jour. Am. Med. Assn.*, 1913, lx, 1141.
[17] Ransom and Kitashima, cited by Zlatogoroff, S. J., *loc. cit.*, p. 694.
[18] Puntoni, V., *Policlinico, sez. med.*, 1913, xx, 385.
[19] Puntoni, V., *Gior. d. r. soc. ital. d'ig.*, 1913, xxxv, 289.
[20] Pottevin, *Bulletin de l'office internationale d'hygiene publique*, 1913, v, 1158.

cultures was seen to disappear or become latent in cultivation, and non-agglutinating vibrios at times apparently spontaneously developed specific agglutination some time after isolation. This observer further states that agglutinating and non-agglutinating vibrios could exist at the same time in one patient.

SUMMARY.

Cholera-like non-agglutinating vibrios are invariably found in the intestinal contents of healthy persons, and frequently in the water of wells and rivers, during epidemics of cholera. Although many of these saprophytic vibrios are indistinguishable in morphology and cultural properties from the cholera vibrio, the negative reaction with an anticholera serum has readily differentiated them from the Asiatic vibrio. The biological polymorphism of the cholera vibrio has been suggested by the development of agglutination, by special methods of culture, in cholera-like vibrios. Confirmatory Pfeiffer reactions have not been obtained, as a rule, in these instances, probably because of the low virulence of the vibrio culture, although positive bacteriolysis *in vitro* (Bordet's test) was observed in some, and in others positive complement fixation and cross agglutination indicated the cholera nature of the vibrios in question.

Although it cannot as yet be definitely proven, we are justified in suspecting that cholera-like vibrios which eventually develop agglutination properties are of a true cholera nature. It is probable that the production of agglutination antibodies in the serum brings about the development by the bacterial cell of defensive anti-agglutinins, resulting in the disappearance of agglutinating power. In the case of the water vibrios, changed physical conditions could bring about a similar alteration in biological properties.

It may be said that the absence of agglutination in a vibrio isolated from a suspected source does not define conclusively its non-cholera nature. In all probability among a number of cholera-like vibrios isolated from suspected sources a certain percentage will eventually be found to develop agglutination either during laboratory cultivation or by means of animal passage, and until subjected to a procedure that will induce the return of agglutination no vibrio can be regarded with assurance as of a truly saprophytic variety.

THE CULTIVATION OF HUMAN TISSUE IN VITRO.*[1]

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Plates 75 to 77.

The present investigations were undertaken to ascertain whether human connective tissue taken from a fresh cadaver could be kept in a condition of permanent life outside of the organism. We have applied to human tissues the method by which Carrel was able to keep animal connective tissue alive *in vitro* for more than two years.[2]

The first attempt to cultivate human tissues *in vitro* was made in 1911 by Carrel and Burrows.[3] Small fragments of human malignant tumors were placed in human plasma and incubated. In a few cases the fragments were surrounded after a few days by many cells; but generally liquefaction of the medium occurred and no growth was observed. In other experiments undertaken later by Carrel on the growth of normal tissues the same phenomenon was observed. Fragments of thyroid gland and fragments of connective tissue from adult individuals or from fetuses were inoculated into culture media which contained or did not contain tissue extract. The liquefaction was observed in this case in the same manner as in the case of the malignant tumors, and with the exception of a few

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[1] These investigations were carried on in March, 1913, in the research laboratory of the New York Lying-In Hospital. They were rendered possible by the coöperation of Dr. Markoe, of the attending surgeons of the Lying-In Hospital, and of Dr. Carrel. The Directors of the Hospital provided a specially equipped laboratory for the work, under the supervision of Dr. Markoe and Dr. Carrel, thus enabling us to carry out the study of the tissues of human cadavers under excellent conditions.

[2] Carrel, A., *Jour. Exper. Med.*, 1913, xviii, 287.

[3] Carrel, A., and Burrows, M. T., *idem*, 1911, xiii, 387.

cultures no real growth was observed. In 1913 Maccabruni[4] cultivated in plasma the tissues of the uterus and ovary of human fetuses and observed around the tissue after 8 or 9 days the presence of many cells and also of karyokinetic figures. The result of these experiments showed that human tissue almost always produced liquefaction of the plasma, and that this probably prevented its growth. We, therefore, attempted to develop a technique which would permit us to keep human tissue in a plasmatic medium without the occurrence of liquefaction.

At first we attempted to obtain a medium which would not liquefy under the influence of the tissue. The first medium tried was composed of human plasma and extract of human tissue taken from fresh cadavers. This, however, proved unsuitable, as in twenty-four hours liquefaction occurred about the fragments of tissue. Very little growth was observed, but when it took place the cells were seen to be scattered and the new growth to be very thin. A thin film containing many cells adhered to the cover-glass. When attempts were made to extirpate the film and to transfer it to fresh medium it generally became folded up and after one or two passages no further growth occurred. Many modifications of the medium were tried, in order to overcome these difficulties. Human plasma was diluted with agar, serum, and egg albumen. The addition of agar and egg albumen to the plasma yielded a firmer medium. No liquefaction took place, but on the other hand no real growth occurred. When the plasma was diluted with serum complete liquefaction rapidly followed. The addition of a small amount of acid or alkali to the plasma did not prevent this liquefaction. Finally after many attempts had been made it was found that by diluting the plasma with equal parts of Ringer solution a medium could be obtained which would not liquefy in less than 24 hours, and often not in 48 and 72 hours. Usually 18 hours after the medium had been inoculated with human tissue, growth appeared and increased progressively. After a period of from 24 to 96 hours the fragments of tissue were transferred to a fresh medium in which the growth continued. The medium was again modified by the addition of a small quantity of diluted extract of human tissue, after which the

[4] Maccabruni, F., *Ann. d. ostet. e ginecol.*, 1914, xxxvi, 57.

growth became very active. Finally at the end of 1913 and during the first months of 1914 it became possible to obtain large growths of human connective tissue. The tissue could be transferred from medium to medium; the new cells could be isolated from a primitive fragment and cultivated in a new medium; the mass of newly formed tissue rapidly increased in the same way that was observed by Carrel to occur in the case of the strain of connective tissue cells taken from the chick embryo, which are still proliferating at The Rockefeller Institute. Nevertheless, the technique which we have developed is still far from perfect. Liquefaction of the medium occurs as soon as the tissue extract becomes too concentrated. Besides, it appears that the plasma taken from many different individuals varies widely, the plasma of certain persons liquefying much more rapidly than that of others. The value of the extracts varies also, the result being that the medium is inconstant; and for this reason the rate of growth of fragments of tissue shows marked fluctuations in the course of a few weeks. The medium will probably be still further modified; nevertheless we shall describe here the technique that has rendered possible the cultivation of human tissue *in vitro*.

TECHNIQUE.

Plasma.—Blood is aspirated from the median basilic vein of adults under aseptic conditions with a dry, chilled, 10 c.c. glass syringe (gauge of needle, 19). The blood is immediately transferred to cold paraffined glass tubes and centrifuged in ice at high speed (3,000 revolutions) for 5 minutes. The plasma is at once pipetted off with a paraffined pipette into another cold paraffined tube, which was kept on ice ready for use. Several paraffined pipettes are prepared with bulbs to be kept in readiness, because it has been found that although the plasma remains liquid for some time in the paraffined tube it coagulates in the pipette used for making the cultures in from two to ten minutes. It is usually necessary to use a fresh pipette after changing two or three cultures.

Extract.—The extract is prepared from tissues taken from adult or fetal cadavers, and cut into small pieces. Equal parts of Ringer solution are added, the whole is placed in cold storage for 48 hours, after which it is centrifuged and the supernatant fluid pipetted off. This fluid may then be kept for some time in cold storage.

Making of Cultures.—Pieces of the various fetal tissues taken from fresh fetal cadavers are placed in Ringer solution or in serum and cut to the proper size with a cataract knife. The plasma is first spread on the cover-glass, and equal parts of Ringer solution are added and thoroughly mixed with it. The object of this

procedure is to obtain a clot which can adhere firmly to the cover-glass, because it was found that when the plasma was diluted with the Ringer solution first coagulation took place too rapidly, thereby preventing a thorough embedding of the tissue. On the other hand, when the Ringer solution is spread first and the plasma added afterwards the clot does not adhere firmly, often contracting to such an extent as to become separated from the cover-glass, thereby destroying the culture. Then the tissue is put into the medium and the extract immediately added. The medium usually coagulates about the piece within a few seconds. A reasonable amount of speed and strict aseptic precautions are observed throughout. The preparations are next placed over a concave slide hermetically sealed with paraffin and incubated at 38° C., and the tissues allowed to grow. When a fresh amount of growth has taken place after a period varying from 24 to 96 hours, the tissues are removed from their medium, washed, and transferred to a fresh medium. The time of the transfer depends upon the condition of the medium and the activity of the growth. Observations are taken at intervals of 12, 24, 48, and even 96 hours. Observations are likewise made of cultures fixed and stained with hematoxylin and with Giemsa stain.

RESULTS.

With the technique described above, it became possible to keep a strain of connective tissue cells, derived from a piece of skin which was obtained from a fresh four months old fetal cadaver, in a condition of active life *in vitro* for more than two months.

When the fragments of fetal heart tissue, obtained from fresh cadavers, were introduced into a medium composed of equal parts of human plasma and Ringer solution, and incubated at 38° C. for 16 hours, microscopic examination of the culture showed the fragments as sharply outlined masses in a slightly opalescent medium with no evidences of cell proliferation. In from 18 to 24 hours cell proliferation manifested itself, in some cases by the appearance of many cells spreading out or budding from the original piece; in other cases by the presence of only a few scattered cells.

The rate and extent of growth varied and probably depended on the vitality of the tissue, the constituents of the medium, and the changes that developed in it. Where there was a tendency for the proliferating cells to grow in one or two planes the growth was greater and more rapid than where the cells appeared to grow in several planes. In the latter case the growth was also more dense.

The phenomenon of growth was accompanied by a progressive and slow liquefaction of the medium around the primitive fragment

and in the zone of cell proliferation. Sometimes liquefaction around the entire fragment took place within 24 hours after the cultures were made, although little, if any, growth was apparent. At times the medium retracted from the piece in certain areas and the vacuoles in the medium thus produced contained fluid in which detached cells and débris were observed. The liquefaction did not indicate necrosis of the piece of tissue in all instances. Pieces around which the medium liquefied completely in 24 hours have been transplanted into fresh medium, in which cell proliferation took place in from 16 to 24 hours. There was no evidence of rapid liquefaction.

When liquefaction was apparently due to cell proliferation, it started from the periphery of the fragment and was progressive. On the other hand, when apparently due to substances in the medium it was general and affected the entire mass of the medium. Liquefaction of the entire medium took place when extract from fetal tissues was used in primitive cultures. Primitive fragments grew well without the addition of extract to the medium for one or two passages, but usually after the second passage it was necessary to add extract in order to stimulate growth. The time of the passages into fresh medium was governed by the condition of the culture. Where the medium showed liquefaction around the primitive piece in 24 hours, a change was made. If the rate of growth was rapid, the culture was changed after 48 hours. Actively growing cultures which were not changed until after 72 hours grew less well. In cases where the time of passage was deferred for 96 hours, subsequent passages showed a decided retardation in the rate and extent of growth of the cells and the cellular characters were altered. Ameboid cells occurred and rapid liquefaction of the entire medium often took place; and although passages into fresh medium were made every 24 or 48 hours the cultures did not recover.

In the history of one culture, which is given in table I, it will be noticed that 24 hours after the 33d passage excellent growth was recorded; 96 hours later the culture was changed, and in 24 hours observation showed retarded cell proliferation with subsequent liquefaction. After the 34th passage, that is, 68 days, there was no further growth and death of the culture gradually resulted. In the

TABLE I.

Passage.	Date (1913).	Treatment of culture.	Observations.
Experiment 121–1	Oct. 21	Culture of skin from a 4 months' fetal cadaver cultivated in 1 drop of plasma and 1 drop of Ringer solution	Oct. 22. Medium in good condition; no growth. Oct. 23. Medium in good condition; growing. Oct. 24. Medium slightly liquefied; growth continuing.
1	Oct. 24	Washed in Ringer solution for 1 minute and cultivated in 1 drop of plasma, 1 drop of Ringer solution, and 1 drop of extract	Oct. 25. Medium liquefied; no growth.
2	Oct. 25	Washed in Ringer solution for 1 minute and cultivated in 1 drop of plasma, 1 drop of Ringer solution, and 1 small drop of extract	Oct. 26. Medium in good condition; growing. Oct. 27. Medium partially liquefied; growing.
3	Oct. 27	Washed in Ringer solution for ½ minute and cultivated in the same medium	Oct. 28. Medium slightly liquefied; growing. Oct. 29. Medium liquefied; growing.
4	Oct. 29	Washed in Ringer solution for ½ minute and cultivated in 2 drops of serum, 1 drop of plasma, and 1 small drop of extract	Oct. 30. Medium completely liquefied; some growth.
5	Oct. 30	Washed in Ringer solution for 1 minute and cultivated in 1 drop of plasma, 1 drop of Ringer solution, and 1 small drop of extract	Oct. 31. Medium in good condition; excellent growth. Nov. 1. Excellent growth.
6	Nov. 1	Divided into 2 pieces, washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 3. Medium in good condition; excellent growth.
7	Nov. 3	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 4. Medium in good condition; excellent growth. Nov. 5. Excellent growth.
8	Nov. 5	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 6. Medium in good condition; excellent growth (macroscopic). Nov. 7. Excellent growth.
9	Nov. 7	Divided into 2 pieces, washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 8. Medium in good condition; growing. Nov. 9. Good growth.
10	Nov. 9	Cut into 2 pieces, washed in Ringer solution for ½ minute, and cultivated in the same medium	Nov. 10. Medium in good condition; excellent growth. Nov. 11. Medium in good condition; excellent growth.
11	Nov. 11	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 12. Medium in good condition; growing well.
12	Nov. 12	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 13. Medium in good condition; good growth.

TABLE I.—*Continued.*

Passage.	Date (1913).	Treatment of culture.	Observations.
13	Nov. 13	Culture washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 14. Growing. Nov. 15. Good growth.
14	Nov. 15	Partially divided, washed in Ringer solution, and cultivated in the same medium	Nov. 17. Good growth.
15	Nov. 17	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 18. Good growth. Nov. 19. Excellent growth.
16	Nov. 19	Washed in Ringer solution for 45 seconds and cultivated in the same medium	Nov. 20. Medium slightly liquefied; growing. Nov. 21. More liquefaction of medium; excellent growth.
17	Nov. 21	Washed in Ringer solution for ½ minute and cultivated in the same medium	Nov. 22. Excellent growth.
18	Nov. 22	Washed in Ringer solution for ½ minute and cultivated in the same medium	Nov. 23. Good growth. Nov. 24. Growing.
19	Nov. 24	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 25. Very good growth. Nov. 26. Liquefaction; excellent growth.
20	Nov. 26	Washed in Ringer solution for 45 seconds and cultivated in the same medium	Nov. 27. Excellent growth. Nov. 28. Liquefaction; excellent growth.
21	Nov. 28	Divided into 2 pieces, washed in Ringer solution for 1 minute, and cultivated in the same medium	Nov. 29. Very good growth.
22	Nov. 29	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 30. Good growth. Dec. 1. Liquefaction; excellent growth.
23	Dec. 1	Washed in Ringer solution for ½ minute and cultivated in the same medium	Dec. 2. Excellent growth (dense).
24	Dec. 2	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 3. Good growth (dense). Dec. 4. Good growth (dense).
25	Dec. 4	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 5. Good growth. Dec. 6. Excellent growth.
26	Dec. 6	Washed in Ringer solution for 45 seconds and cultivated in the same medium	Dec. 7. Growing slowly. Dec. 8. Liquefaction and growing slowly. All cultures this day liquefied.
27	Dec. 8	Washed in Ringer solution for 45 seconds and cultivated in the same medium	Dec. 9. Growing. Dec. 10. Slight liquefaction; growing.
28	Dec. 10	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 11. Growing. Dec. 12. Growing.
29	Dec. 12	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 14. Good growth. Dec. 15. Excellent growth.

TABLE I.—*Concluded.*

Passage.	Date (1913).	Treatment of culture.	Observations.
30	Dec. 15	Central piece extirpated and the remaining cells cultivated in 1 drop of plasma and 1 drop of extract	Dec. 16. Excellent growth. Dec. 17. Excellent growth.
31	Dec. 17	Washed in Ringer solution and cultivated in 1 drop of plasma, 1 drop of Ringer solution, and 1 drop of extract	Dec. 18. Growing very well. Dec. 19. Growing very well.
32	Dec. 19	Divided into 2 pieces, washed in Ringer solution for 1 minute, and cultivated in the same medium	Dec. 20. Liquefaction; growing. Dec. 21. Liquefaction; excellent growth.
33	Dec. 22	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 22. Excellent growth. Dec. 23. Growing. Dec. 24. Medium in good condition; no liquefaction. Dec. 25. Medium in good condition; no liquefaction. Dec. 26. Medium in good condition; no liquefaction.
34	Dec. 26	Divided into 2 pieces, washed in Ringer solution for 1 minute, and cultivated in the same medium	Dec. 27. Growing slowly. Dec. 28. Growing slowly. Dec. 29. Liquefaction; growing slowly.
35	Dec. 29	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 30. No growth; ameboid cells. Dec. 31. Complete liquefaction.
36	Dec. 31	Culture washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 2, 1914. Few cells, mostly ameboid in character.

first and succeeding passages growth was more extensive in a given time than that which develops in a primitive culture; also a definite relation between the extent of growth and the degree of liquefaction which is produced by the growth was suggested.

Fluctuations were observed in the rate and extent of growth after passages, a point which is shown in the history referred to. This fluctuation affected all cultures which were changed at the same time and into the same medium. The probable causes are the differences in various specimens of plasma and the effect of the extract employed on them. In successive passages the primitive fragment gradually disappeared; and on extirpation of the primitive fragment the peripheral cells after being washed and cultivated in fresh medium continued to proliferate and form a network of tissue as shown in figure 1. Figure 2 shows the extirpated fragment which

was washed and cultivated and incubated for forty-eight hours, after which it was stained.

In actively growing cultures the cells appeared as delicate spindle-shaped, fusiform bodies, isolated or united by filaments sometimes closely assembled, forming a network which was either film-like or quite dense, depending upon the tendency of the cellular outgrowths. The cytoplasm appeared granular and the nucleus stood out as a clear, homogeneous area in which mitotic figures were distinguishable. Refractile globules were to be seen in the cytoplasm of the cells. Their size and number varied. The longer a culture was left unchanged the larger and more numerous were these globules. When conditions in the culture were unfavorable, the cells presented ameboid characteristics, and there was no tendency towards the formation of a cellular network. Refractile fat globules then became frequent in the cell cytoplasm.

When the cultures were fixed and stained with Giemsa stain the outline and structure of the cells became distinct. The cytoplasm appeared as a light blue, finely granular body with a more deeply stained, coarsely granular nucleus. The structures containing chromatin took on a deep purple stain. Figure 3 represents a photograph of stained individual cells highly magnified.

Although growth of human connective tissue derived from fetal heart tissue has been obtained which was as extensive as the growth of connective tissue derived from embryonic heart tissue of the chick, the growth of human tissue was usually less dense and less extensive. Fluctuations which occurred during the passages of human cultures were greater than those occurring in cultures of chick tissue. Liquefaction in human tissue cultures was more marked and eventually interfered with cell proliferation. In cultures of chick tissue the liquefaction that took place was slight and did not interfere with cell proliferation.

The fetal human tissue which was used in making cultures was generally obtained from fetal cadavers 4 to 6 months old; the embryonic chick tissue was obtained from 8 to 15 day old embryos. The latent period where human tissue was cultivated was usually from 16 to 18 hours. For chick tissue growth appears within 10 or 12 hours after the original fragment has been embedded.

SUMMARY.

A strain of human connective tissue was kept in a condition of active life *in vitro* for more than two months. When a medium has been devised the composition of which is more constant, it is reasonable to suppose that human connective tissue can be cultivated *in vitro* for an indefinite period.

EXPLANATION OF PLATES.

PLATE 75.

FIG. 1. Human connective tissue cells, fixed and stained with Giemsa stain. The culture was made by extirpating the central portion of culture 285 in its 16th passage, washing the remaining portion of the culture with Ringer solution without removing it from the cover-glass, and dropping on fresh plasma and extract. The preparation shows the extent of growth obtained in 48 hours from peripheral cells remaining after extirpation of the fragment.

PLATE 76.

FIG. 2. 17th passage of human connective tissue, fixed and stained with Giemsa stain. The photograph shows the extent of growth obtained after passage into fresh medium. The fragment in this culture was the piece extirpated from culture 285.

PLATE 77.

FIG. 3. 17th passage of human connective tissue cells. Individual cells; high power magnification. Some of the cells are shown in figure 2.

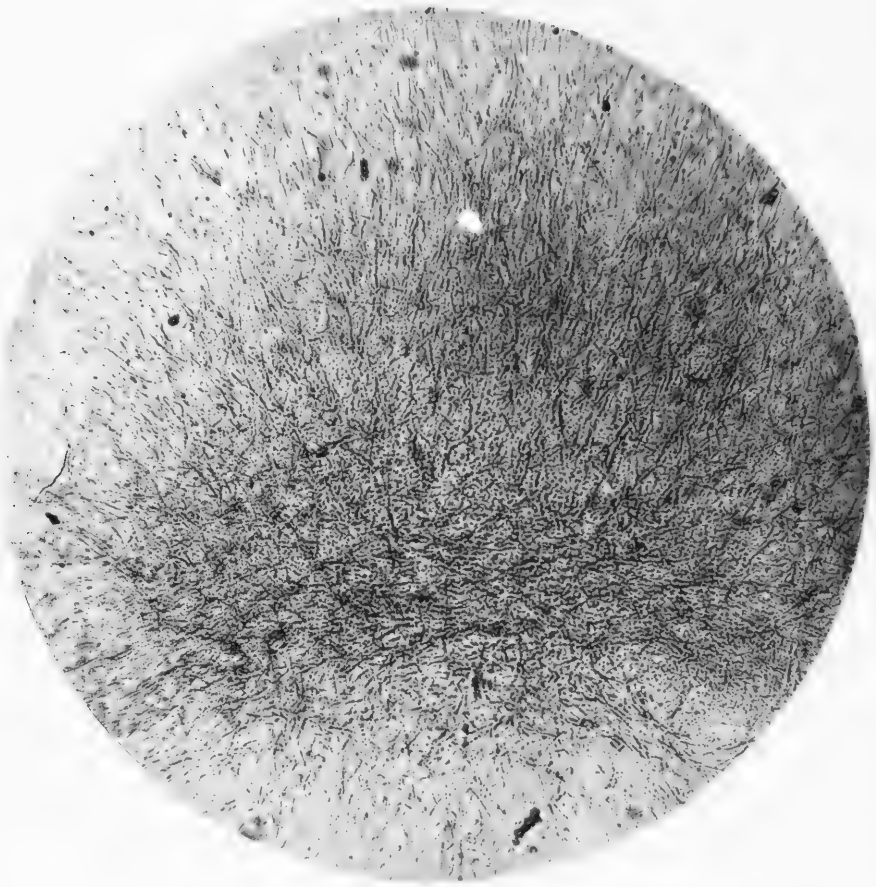

FIG. 1.

Losee and Ebeling: The Cultivation of Human Tissue *in vitro*.)

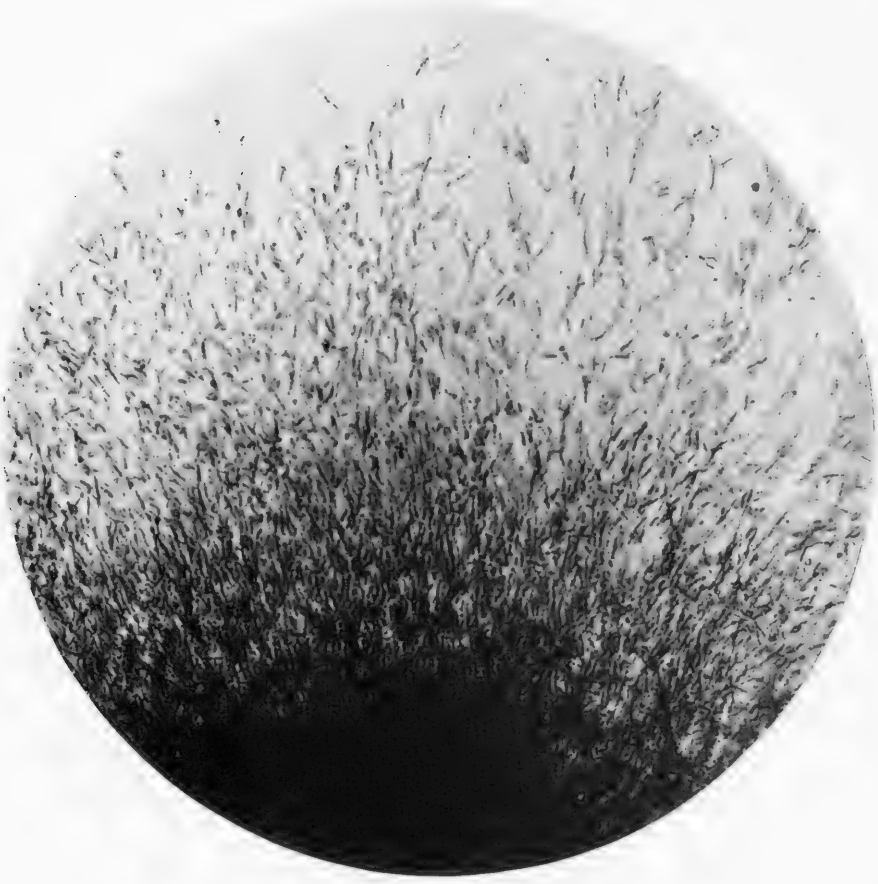

FIG. 2.

(Losee and Ebeling: The Cultivation of Human Tissue *in vitro*.)

FIG. 3.

(Losee and Ebeling: The Cultivation of Human Tissue *in vitro*.)

INDEX TO VOLUME XIX.

ABSORPTION, mechanism of, from the mucosa of closed duodenal loops, in intestinal obstruction, 166
Acid agglutination of pneumococci, 28
Adrenalin index of the suprarenal glands in health and disease, 536
Agglutination, acid, of pneumococci, 28
AMOSS, HAROLD L. A note on the etiology of epidemic poliomyelitis, 212
——. See CLARK and AMOSS, 217
——. See CLARK, FRASER, and AMOSS, 273
——. See FLEXNER and AMOSS, 411
——. See FLEXNER, CLARK, and AMOSS, 195, 205
Anesthetic in animal experimentation, nitrous oxide as an, 372
Anilin dyes, growth of bacteria on media containing, with special reference to an enrichment method for typhoid and paratyphoid bacilli, 501
Anilin dyes, observations on the growth of bacteria on media containing, 20
Antitrypsin, serum, nature of, 459
Arteriosus, ductus, (Botalli), in *Sus scrofa*, behavior of elastic tissue in postfetal occlusion and obliteration of, 129
Arthritis, experimental, in the rabbit, produced with *Streptococcus mitis*, 444
Aschoff, nodules of, submiliary myocardial, in rheumatic fever, significance of, 417

BACILLI, tubercle, ferment-inhibiting substances in, 251
Bacilli, typhoid and paratyphoid, enrichment method for, and the growth of bacteria on media containing various anilin dyes, 501
Bacillus tuberculosis, function of spleen in experimental infection of albino mice with, 187
Bacteria, growth of, on media containing various anilin dyes, with special reference to an enrichment method for typhoid and paratyphoid bacilli, 501
Bacteria, observations on growth of, on media containing various anilin dyes, 20
BERNHEIM, B. M. See WHIPPLE, STONE, and BERNHEIM, 144, 166
Bile, fat content of, and fatty changes in the liver, relation between, 234
Blood, penetration of the virus of poliomyelitis from, into the cerebrospinal fluid, 411
Blood pressure variations, human, and cardiac rhythm, 1
Blood, relation to, of the virus of epidemic poliomyelitis, 223
Blood, retention of nitrogen in, in experimental acute nephritis, 259
Blood sinuses, spindle-celled sarcomata rifted with, 570
BOWMAN, FRED B. See EVANS, BOWMAN, and WINTERNITZ, 283
BROWN, WADE H. A note on the pathogenicity of *Trypanosoma lewisi*, 406
——. Morphological and developmental anomalies of a pathogenic strain of *Trypanosoma lewisi* and their relation to its virulence, 562

CALCIUM salts, effect of; relation of the parathyroids to the thyroid and to sugar tolerance; relation of tetany to age, amount of parathyroid tissue removed, accessory parathyroids, pregnancy, lactation, rickets, sulphur, and diet, 89
Cardiac rhythm and human blood pressure variations, 1
CARY, EDWARD G. See ZINSSER and CARY, 345
Cells in tissue cultures, effect of dilution of plasma medium on the growth and fat accumulation of, 398
Cerebrospinal fluid, penetration of the virus of poliomyelitis into, from the blood, 411
Cholera vibrio, recognition of, 581
CLARK, PAUL F., and AMOSS, HAROLD L. Intraspinous infection in experimental poliomyelitis, 217
CLARK, PAUL F., FRASER, FRANCIS R., and AMOSS, HAROLD L. The rela-

tion to the blood of the virus of epidemic poliomyelitis, 223
CLARK, PAUL F. See FLEXNER, CLARK, and AMOSS, 195, 205
Corpuscle, red, evidence of the extracellular relation of the parasite to. A stage in the migration of the adult tertian malarial parasite, 450
CRASTER, C. V. The recognition of the cholera vibrio, 581
Cultivation of human tissue *in vitro*, 593
Cultures of tissue, effect of dilution of plasma medium on the growth and fat accumulation of cells in, 398
Cytotoxins, specificity of, 277

DELATOUR, B. J. See SYDENSTRICKER, DELATOUR, and WHIPPLE, 536
Diet, sulphur, pregnancy, lactation, rickets, age, amount of parathyroid tissue removed, and accessory parathyroids, relation of tetany to; relation of parathyroids to thyroid and to sugar tolerance; effect of calcium salts, 89
DOLLEY, DAVID H. A note on nitrous oxide as an anesthetic in animal experimentation, 372
Ductus arteriosus (Botalli) in *Sus scrofa*, behavior of elastic tissue in postfetal occlusion and obliteration of the, 129
Duodenal loop poison, the defensive mechanism of the immunized animal against, in intestinal obstruction, 144
Duodenal loops, closed, in intestinal obstruction, mechanism of absorption from the mucosa of, 166
Dyes, anilin, growth of bacteria on media containing, with special reference to an enrichment method for typhoid and paratyphoid bacilli, 501
Dyes, anilin, observations on the growth of bacteria on media containing, 20

EBELING, ALBERT H. See LOSEE and EBELING, 593
Elastic tissue, the behavior of, in the postfetal occlusion and obliteration of the ductus arteriosus (Botalli) in *Sus scrofa*, 129
Embryo of chick, the ultimate fate of mammalian tissue implanted in, 181
Epidemic poliomyelitis, a note on the etiology of, 212
Epidemic poliomyelitis, contribution to the pathology of, 205
Epidemic poliomyelitis, relation of the virus of, to the blood, 223
Epidemiology of poliomyelitis, contribution to, 195
Etiology of epidemic poliomyelitis, a note on, 212
EVANS, HERBERT M., BOWMAN, FRED B., and WINTERNITZ, M. C. An experimental study of the histogenesis of the miliary tubercle in vitally stained rabbits, 283
Experimental acute nephritis, nitrogen retention in the blood in, 259
Experimental arthritis in the rabbit, produced with *Streptococcus mitis*, 444
Experimental focalized myocardial lesions produced with *Streptococcus mitis*, 429
Experimental hydrocephalus, 106
Experimental infection of albino mice with *Bacillus tuberculosis*, function of the spleen in, 187
Experimental poliomyelitis, intraspinous infection in, 217
Experimental study of the histogenesis of the miliary tubercle in vitally stained rabbits, 283
Experimentation, animal, nitrous oxide as an anesthetic in, 372

FAT accumulation and growth of cells in tissue cultures, effect of dilution of plasma medium on, 398
Fat content of the bile and fatty changes in the liver, relation between, 234
Ferment action, studies on, 239, 251, 383, 459, 480
Ferment-inhibiting agents, soaps as, 239
Ferment-inhibiting substances and ferments in tuberculous caseous material, 383
Ferment-inhibiting substances in tubercle bacilli, 251
Ferments and ferment-inhibiting substances in tuberculous caseous material, 383
Fever, rheumatic, significance of submiliary myocardial nodules of Aschoff in, 417
Filterable agents, causation by, of three distinct chicken tumors, 52
FLEXNER, SIMON, and AMOSS, HAROLD L. Penetration of the virus of poliomyelitis from the blood into the cerebrospinal fluid, 411
FLEXNER, SIMON, CLARK, PAUL F., and AMOSS, HAROLD L. A contribution to the epidemiology of poliomyelitis, 195

——. A contribution to the pathology of epidemic poliomyelitis, 205
Fluid, cerebrospinal, penetration of the virus of poliomyelitis into, from the blood, 411
Focalized myocardial lesions, experimental, produced with *Streptococcus mitis*, 429
FRASER, FRANCIS R. See CLARK, FRASER, and AMOSS, 223

GASTRO-INTESTINAL tract and contents, relation of, to the body-weight in rabbits, 339
GILLESPIE, L. J. The acid agglutination of pneumococci, 28
Glands, suprarenal, adrenalin index of, in health and disease, 536
Goitre (so called thyroid carcinoma) in brook trout (*Salvelinus fontinalis*), further observations and experiments on. III. Its prevention and cure, 70
Grafting of tissue, heteroplastic, factors of resistance to, 513

HANES, FREDERIC M. An immunological study of *Pneumococcus mucosus*, 38
Heteroplastic tissue-grafting, factors of resistance to, 513
Histogenesis of the miliary tubercle in vitally stained rabbits, an experimental study of, 283
Hydrocephalus, experimental, 106
Hyperplasias, active thyroid, of brook trout, rapidity of the involution of, following the use of fresh sea fish as a food, 376

IMMUNIZED animal, the defensive mechanism of the, against duodenal loop poison, in intestinal obstruction, 144
Immunological study of *Pneumococcus mucosus*, 38
In vitro cultivation of human tissue, 593
Indophenoloxydase content of tissues from rabbits infected with pneumococcus, 303
Infection, experimental, of albino mice with *Bacillus tuberculosis*, function of the spleen in, 187
Infection, intraspinous, in experimental poliomyelitis, 217
Infection, ovarian, in the domestic fowl and direct transmission of disease to the offspring, 552
Infections, tertian malarial, effect of the migration of the parasites of, and free malarial parasites, 523
Intestinal obstruction, 144, 166
Intraspinous infection in experimental poliomyelitis, 217

JOBLING, JAMES W., and PETERSEN, WILLIAM. Soaps as ferment-inhibiting agents. Studies on ferment action. X, 239
——. Ferment-inhibiting substances in tubercle bacilli. Studies on ferment action. XI, 251
——. A study of the ferments and ferment-inhibiting substances in tuberculous caseous material. Studies on ferment action. XII, 383
——. The nature of serum antitrypsin. Studies on ferment action. XIII, 459
——. Serotoxin. Studies on ferment action. XIV, 480

KARSNER, HOWARD T., and DENIS, W. A further study of nitrogen retention in the blood in experimental acute nephritis, 259
——. A note on nitrogen retention following repeated injections of nephrotoxic agents, 270
KRUMWIEDE, CHARLES, JR., and PRATT, JOSEPHINE S. Observations on the growth of bacteria on media containing various anilin dyes, 20
——. Further observations on the growth of bacteria on media containing various anilin dyes, with special reference to an enrichment method for typhoid and paratyphoid bacilli, 501

LACTATION, pregnancy, rickets, sulphur, diet, age, amount of parathyroid tissue removed, and accessory parathyroids, relation of tetany to; relation of parathyroids to the thyroid and to sugar tolerance; effect of calcium salts, 89
LAMBERT, ROBERT A. A note on the specificity of cytotoxins, 277
——. The effect of dilution of plasma medium on the growth and fat accumulation of cells in tissue cultures, 398
LANGE, LINDA B. On certain spontaneous chicken tumors as manifestations of a single disease. II. Simple spindle-celled sarcomata, 577
LAWSON, MARY R. A stage in the migration of the adult tertian malarial parasite. Evidence of the extracellular relation of the parasite to the red corpuscle, 450
——. Free malarial parasites and the

effect of the migration of the parasites of tertian malarial infections, 523
Le Count, Edwin R., and Long, Esmond R. The relation between the fat content of the bile and fatty changes in the liver, 234
Lesions, experimental focalized myocardial, produced with *Streptococcus mitis*, 429
Lewis, Paul A., and Margot, Arthur Georges. The function of the spleen in the experimental infection of albino mice with *Bacillus tuberculosis*, 187
Lewisi, Trypanosoma, a note on the pathogenicity of, 406
Lewisi, Trypanosoma, morphological and developmental anomalies of a pathogenic strain of, and their relation to its virulence, 562
Liver, fatty changes in, and fat content of the bile, relation between, 234
Livingston, A. E. The relation of the gastro-intestinal tract and contents to the body-weight in rabbits, 339
Long, Esmond R. See Le Count and Long, 234
Losee, Joseph R., and Ebeling, Albert H. The cultivation of human tissue *in vitro*, 593

Malarial parasite, adult tertian, a stage in the migration of. Evidence of the extracellular relation of the parasite to the red corpuscle, 450
Malarial parasites, free, and the effect of the migration of the parasites of tertian malarial infections, 523
Mammalian tissue, adult, survival and transplantability of, in simple plasma, 121
Mammalian tissue implanted in the chick embryo, ultimate fate of, 181
Margot, Arthur Georges. See Lewis and Margot, 187
Marine, David. Further observations and experiments on goitre (so called thyroid carcinoma) in brook trout (*Salvelinus fontinalis*). III. Its prevention and cure, 70
——. Observations on tetany in dogs. Relation of the parathyroids to the thyroid; relation of tetany to age, amount of parathyroid tissue removed, accessory parathyroids, pregnancy, lactation, rickets, sulphur, and diet; relation of parathyroids to sugar tolerance; effect of calcium salts, 89
——. The rapidity of the involution of active thyroid hyperplasias of brook trout following the use of fresh sea fish as a food, 376
Media containing various anilin dyes, growth of bacteria on, with special reference to an enrichment method for typhoid and paratyphoid bacilli, 501
Media containing various anilin dyes, observations on growth of bacteria on, 20
Medigreceanu, Florentin. The indophenoloxydase content of tissues from rabbits infected with pneumococcus, 303
——. A comparative study of the rate of proteolysis of tissues obtained from rabbits infected with pneumococcus, 309
Medium, plasma, effect of dilution of, on the growth and fat accumulation of cells in tissue cultures, 398
Miliary tubercle in vitally stained rabbits, an experimental study of the histogenesis of, 283
Mitis, Streptococcus, experimental arthritis in the rabbit produced with, 444
Mitis, Streptococcus, experimental focalized myocardial lesions produced with, 429
Morphological and developmental anomalies of a pathogenic strain of *Trypanosoma lewisi* and their relation to its virulence, 562
Mucosa of closed duodenal loops in intestinal obstruction, mechanism of absorption from, 166
Mucosus, Pneumococcus, an immunological study of, 38
Murphy, James B. Studies in tissue specificity. II. The ultimate fate of mammalian tissue implanted in the chick embryo, 181
——. Factors of resistance to heteroplastic tissue-grafting. Studies in tissue specificity. III, 513
——. See Rous and Murphy, 52
Myocardial lesions, focalized, experimental, produced with *Streptococcus mitis*, 429
Myocardial nodules of Aschoff, submiliary, in rheumatic fever, significance of, 417

Nephritis, acute experimental, nitrogen retention in the blood in, 259
Nephrotoxic agents, nitrogen retention following repeated injections of, 270

Nervous system, observations on a strain of *Spirochæta pallida* isolated from, 362
NICHOLS, HENRY J. Observations on a strain of *Spirochæta pallida* isolated from the nervous system, 362
Nitrogen retention following repeated injections of nephrotoxic agents, 270
Nitrogen retention in the blood in experimental acute nephritis, 259
Nitrous oxide as an anesthetic in animal experimentation, 372
Nodules of Aschoff, submiliary myocardial, in rheumatic fever, significance of, 417

OPSONIC substances of normal sera, on the nature of, 345
Ovarian infection in the domestic fowl and direct transmission of disease to the offspring, 552
Oxide, nitrous, as an anesthetic in animal experimentation, 372

PALLIDA, *Spirochæta*, isolated from the nervous system, observations on a strain of, 362
PAPPENHEIMER, ALWIN M. The effects of early extirpation of the thymus in albino rats, 319
Parasite, adult tertian malarial, a stage in the migration of. Evidence of the extracellular relation of the parasite to the red corpuscle, 450
Parasites, free malarial, and the effect of the migration of the parasites of tertian malarial infections, 523
Parathyroid tissue removed, age, accessory parathyroids, pregnancy, lactation, rickets, sulphur, and diet, relation of tetany to; relation of parathyroids to the thyroid and to sugar tolerance; effect of calcium salts, 89
Parathyroids, relation of, to the thyroid and to sugar tolerance in tetany in dogs. Relation of tetany to age, amount of parathyroid tissue removed, accessory parathyroids, pregnancy, lactation, rickets, sulphur, and diet; effect of calcium salts, 89
Paratyphoid and typhoid bacilli, enrichment method for, and the growth of bacteria on media containing various anilin dyes, 501
Pathogenic strain of *Trypanosoma lewisi*, morphological and developmental anomalies of, and their relation to its virulence, 562
Pathogenicity of *Trypanosoma lewisi*, 406
Pathology of epidemic poliomyelitis, contribution to, 205
Penetration of the virus of poliomyelitis from the blood into the cerebrospinal fluid, 411
PETERSEN, WILLIAM. See JOBLING and PETERSEN, 239, 251, 383, 459, 480
Plasma medium, effect of dilution of, on the growth and fat accumulation of cells in tissue cultures, 398
Plasma, simple, survival and transplantability of adult mammalian tissue in, 121
Pneumococci, acid agglutination of, 28
Pneumococci, tissues obtained from rabbits infected with, and tissues from normal rabbits, comparative study of the rate of proteolysis of, 309
Pneumococcus, indophenoloxydase content of tissues from rabbits infected with, 303
Pneumococcus mucosus, an immunological study of, 38
Poliomyelitis, contribution to the epidemiology of, 195
Poliomyelitis, epidemic, a note on the etiology of, 212
Poliomyelitis, epidemic, contribution to the pathology of, 205
Poliomyelitis, epidemic, relation of the virus of, to the blood, 223
Poliomyelitis, experimental, intraspinous infection in, 217
Poliomyelitis, penetration of the virus of, from the blood into the cerebrospinal fluid, 411
Postfetal occlusion and obliteration of the ductus arteriosus (Botalli) in *Sus scrofa*, behavior of elastic tissue in, 129
PRATT, JOSEPHINE S. See KRUMWIEDE and PRATT, 20, 501
Pregnancy, amount of parathyroid tissue removed, age, lactation, rickets, sulphur, diet, and accessory parathyroids, relation of tetany to; relation of the parathyroids to the thyroid and to sugar tolerance; effect of calcium salts, 89
Proteolysis of tissues obtained from rabbits infected with pneumococci and of tissues from normal rabbits, comparative study of the rate of, 309

RED corpuscle, evidence of the extracellular relation of the parasite to, a stage in the migration of the adult tertian malarial parasite, 450
RETTGER, LEO F. Ovarian infection in

the domestic fowl and direct transmission of disease to the offspring, 552
Rheumatic fever, significance of submiliary myocardial nodules of Aschoff in, 417
Rickets, pregnancy, lactation, sulphur, diet, age, amount of parathyroid tissue removed, and accessory parathyroids, relation of tetany to; relation of parathyroids to the thyroid and to sugar tolerance; effect of calcium salts, 89
ROTHSCHILD, M. A., and THALHIMER, WILLIAM. Experimental arthritis in the rabbit, produced with *Streptococcus mitis*, 444
ROTHSCHILD, M. A. See THALHIMER and ROTHSCHILD, 417, 429
ROUS, PEYTON. On certain spontaneous chicken tumors as manifestations of a single disease. I. Spindle-celled sarcomata rifted with blood sinuses, 570
ROUS, PEYTON, and MURPHY, JAMES B. On the causation by filterable agents of three distinct chicken tumors, 52

SALTS, calcium, effect of; relation of the parathyroids to the thyroid and to sugar tolerance; relation of tetany to age, amount of parathyroid tissue removed, accessory parathyroids, pregnancy, lactation, rickets, sulphur, and diet, 89
Sarcomata, simple spindle-celled, 577
Sarcomata, spindle-celled, rifted with blood sinuses, 570
SCHAEFFER, J. PARSONS. The behavior of elastic tissue in the postfetal occlusion and obliteration of the ductus arteriosus (Botalli) in *Sus scrofa*, 129
Sera, normal, on the nature of the opsonic substances of, 345
Serotoxin, 480
Serum antitrypsin, nature of, 459
Sinuses, blood, spindle-celled sarcomata rifted with, 570
Soaps as ferment-inhibiting agents, 239
Specificity of cytotoxins, 277
Specificity of tissue, studies in, 181, 513
Spindle-celled sarcomata rifted with blood sinuses, 570
Spindle-celled sarcomata, simple, 577
Spirochæta pallida isolated from the nervous system, observations on a strain of, 362
Spleen, function of, in the experimental infection of albino mice with *Bacillus tuberculosis*, 187
STONE, H. B. See WHIPPLE, STONE, and BERNHEIM, 144, 166
Streptococcus mitis, experimental arthritis in the rabbit produced with, 444
Streptococcus mitis, experimental focalized myocardial lesions produced with, 429
Submiliary myocardial nodules of Aschoff in rheumatic fever, significance of, 417
Sugar tolerance, relation of parathyroids to; effect of calcium salts; relation of the parathyroids to the thyroid; relation of tetany to age, amount of parathyroid tissue removed, accessory parathyroids, pregnancy, lactation, rickets, sulphur, and diet, 89
Sulphur, diet, pregnancy, lactation, rickets, age, amount of parathyroid tissue removed, and accessory parathyroids, relation of tetany to; relation of parathyroids to thyroid and to sugar tolerance; effect of calcium salts, 89
Suprarenal glands, adrenalin index of, in health and disease, 536
SYDENSTRICKER, V. P. W., DELATOUR, B. J., and WHIPPLE, G. H. The adrenalin index of the suprarenal glands in health and disease, 536

TERTIAN malarial infections, effect of the migration of the parasites of, and free malarial parasites, 523
Tertian malarial parasite, adult, a stage in the migration of. Evidence of the extracellular relation of the parasite to the red corpuscle, 450
Tetany in dogs, observations on. Relation of the parathyroids to the thyroid; relation of tetany to age, amount of parathyroid tissue removed, accessory parathyroids, pregnancy, lactation, rickets, sulphur, and diet; relation of parathyroids to sugar tolerance; effect of calcium salts, 89
THALHIMER, WILLIAM, and ROTHSCHILD, M. A. On the significance of the submiliary nodules of Aschoff in rheumatic fever, 417
——. Experimental focalized myocardial lesions produced with *Streptococcus mitis*, 429
THALHIMER, WILLIAM. See ROTHSCHILD and THALHIMER, 444
THOMAS, WALTER S. Experimental hydrocephalus, 106

Thymus in albino rats, effects of early extirpation of, 319
Thyroid carcinoma, so called, in brook trout (*Salvelinus fontinalis*), further observations and experiments on. III. Its prevention and cure, 70
Thyroid hyperplasias, active, of brook trout, rapidity of the involution of, following the use of fresh sea fish as a food, 376
Thyroid, relation of the parathyroids to; relation of tetany to age, amount of parathyroid tissue removed, accessory parathyroids, pregnancy, lactation, rickets, sulphur, and diet; relation of parathyroids to sugar tolerance; effect of calcium salts, 89
Tissue, adult mammalian, survival and transplantability of, in simple plasma, 121
Tissue cultures, cells in, effect of dilution of plasma medium on the growth and fat accumulation of, 398
Tissue, elastic, the behavior of, in the postfetal occlusion and obliteration of the ductus arteriosus (Botalli) in *Sus scrofa*, 129
Tissue-grafting, heteroplastic, factors of resistance to, 513
Tissue, human, cultivation of, *in vitro*, 593
Tissue, mammalian, implanted in the chick embryo, ultimate fate of, 181
Tissue of parathyroid, removed, accessory parathyroids, pregnancy, lactation, rickets, sulphur, diet, and age, relation of tetany to; relation of parathyroids to the thyroid and to sugar tolerance; effect of calcium salts, 89
Tissue specificity, studies in, 181, 513
Tissues from rabbits infected with pneumococcus, indophenoloxydase content of, 303
Tissues obtained from rabbits infected with pneumococci and tissues from normal rabbits, comparative study of the rate of proteolysis of, 309
Transplantability and survival of adult mammalian tissue in simple plasma, 121
Trypanosoma lewisi, a note on the pathogenicity of, 406
Trypanosoma lewisi, morphological and developmental anomalies of a pathogenic strain of, and their relation to its virulence, 562
Tubercle bacilli, ferment-inhibiting substances in, 251
Tubercle, miliary, in vitally stained rabbits, an experimental study of the histogenesis of, 283
Tuberculosis, Bacillus, function of the spleen in experimental infection of albino mice with, 187
Tuberculous caseous material, study of the ferments and ferment-inhibiting substances in, 383
Tumors of the chicken, spontaneous, as manifestations of a single disease, 570, 577
Tumors of the chicken, three distinct, causation of, by filterable agents, 52
Typhoid and paratyphoid bacilli, enrichment method for, and the growth of bacteria on media containing various anilin dyes, 501

VIBRIO of cholera, recognition of, 581
Virus of epidemic poliomyelitis, relation of, to the blood, 223
Virus of poliomyelitis, penetration of, from the blood into the cerebrospinal fluid, 411

WALTON, ALBERT J. On the survival and transplantability of adult mammalian tissue in simple plasma, 121
WHIPPLE, G. H., STONE, H. B., and BERNHEIM, B.M. Intestinal obstruction. III. The defensive mechanism of the immunized animal against duodenal loop poison, 144
——. Intestinal obstruction. IV. The mechanism of absorption from the mucosa of closed duodenal loops, 166
WHIPPLE, G. H. See SYDENSTRICKER, DELATOUR, and WHIPPLE, 536
WIGGERS, CARL J. Does cardiac rhythm alone determine human blood pressure variations?, 1
WINTERNITZ, M. C. See EVANS, BOWMAN, and WINTERNITZ, 283

ZINSSER, HANS, and CARY, EDWARD G. On the nature of the opsonic substances of normal sera, 345

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CONTENTS.

LAWSON, MARY R. Free Malarial Parasites and the Effect of the Migration of the Parasites of Tertian Malarial Infections. Plates 61 to 66 523

SYDENSTRICKER, V. P. W., DELATOUR, B. J., and WHIPPLE, G. H. The Adrenalin Index of the Suprarenal Glands in Health and Disease 536

RETTGER, LEO F. Ovarian Infection in the Domestic Fowl and Direct Transmission of Disease to the Offspring . . . 552

BROWN, WADE H Morphological and Developmental Anomalies of a Pathogenic Strain of *Trypanosoma lewisi* and Their Relation to Its Virulence. Plates 67 and 68 562

ROUS, PEYTON. On Certain Spontaneous Chicken Tumors as Manifestations of a Single Disease. I. Spindle-Celled Sarcomata Rifted with Blood Sinuses. Plates 69 to 71 . . 570

LANGE, LINDA B. On Certain Spontaneous Chicken Tumors as Manifestations of a Single Disease. II. Simple Spindle-Celled Sarcomata. Plates 72 to 74 577

CRASTER, C. V. The Recognition of the Cholera Vibrio . . 581

LOSEE, JOSEPH R., and EBELING, ALBERT H. The Cultivation of Human Tissue *in Vitro*. Plates 75 to 77 593

Index to Volume XIX 603

The Journal of Experimental Medicine is issued monthly, two volumes appearing in a year. Each volume contains more than 600 pages and many plates and text-figures. Subscriptions will not be accepted to begin in the middle of a volume. Subscribers should state whether they wish their subscription to begin with the January or July volume, unless they wish to leave the decision to the option of the publishers.

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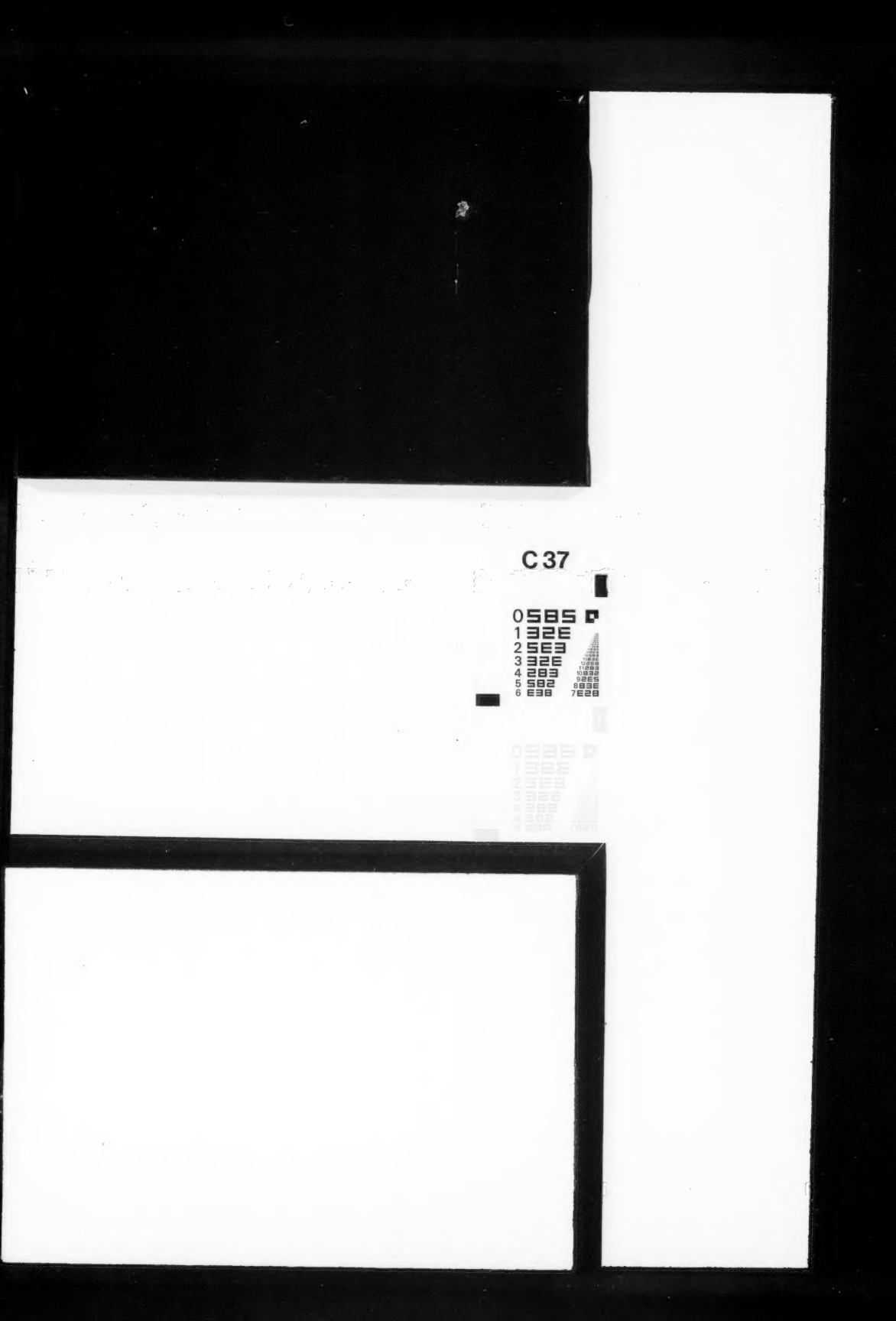
C37
0585
1 32E
2 5E3
3 32E
4 283
5 582
6 E38